

ORIGINAL ARTICLE

CD4⁺CD25⁺ T regulatory cells do not transfer oral tolerance to peanut allergens in a mouse model of peanut allergy

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Clinical & Experimental Allergy

Summary

Background Recent studies have implicated CD4⁺CD25⁺ regulatory T cells (nTregs) in the maintenance of tolerance to oral antigens and in the regulation of the food allergic IgE response.

Objective The objective was to assess if nTregs can transfer allergen-specific oral tolerance to naïve, non-TCR transgenic mice and regulate peanut extract (PE)-specific hypersensitivity responses. Additionally, the role of the regulatory cytokines IL-10 and TGF-β in the modulation of peanut-allergic sensitization was studied.

Methods CD25-enriched T cells from PE-tolerant mice were adoptively transferred to recipient mice, which were subsequently sensitized to PE. Depletion of CD25⁺ cells and neutralization of IL-10 and TGF-β were compared in a CH3/HeOuJ mouse model of peanut-allergic sensitization.

Results Transfer of CD25⁺ Tregs-enriched cell populations did not affect the PE-specific cytokine production or PE-specific antibody levels compared with control mice but interestingly resulted in a decrease of mast cell responsiveness. On the contrary, transfer of CD25⁺ Tregs-depleted cells caused an increase in non-specific cytokine production, in the absence of changes in PE-specific responses. TGF-β neutralization resulted even in a larger increase in spontaneous release of all cytokines measured (IL-4, IL-5, IL-10, IL-13, and IFN-γ), but surprisingly also to a higher PE-specific Th2-associated (IL-4, IL-5, IL-13) cytokine production compared with depletion of CD25 cells or neutralization of IL-10. Similarly, depletion of CD25 cells and TGF-β neutralization but not of IL-10 neutralization lead to an increase in PE-specific antibody levels and elevated mast cell degranulation following a PE challenge.

Conclusions and Clinical Relevance We conclude that CD4⁺CD25⁺ Tregs from non-transgenic-tolerant mice cannot transfer specific oral tolerance of exogenous antigens to naïve mice and are more involved in general immune suppressive mechanisms. However, we found evidence that TGF-β secreting Tregs (Th3) may play an important role.

Keywords food allergy, oral tolerance, peanuts, sensitization

Submitted 26 October 2009; revised 1 September 2010; accepted 29 September 2010

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Introduction

It is estimated that currently 3–4% of adult individuals are suffering from some kind of food allergy [1]. Peanut-allergy accounts for the majority of severe food-related allergic reactions and is most commonly associated with anaphylaxis and a fatal outcome [2]. In addition, peanut allergy does not usually resolve, resulting in a life-long allergy with a high impact on daily life. So far, no therapies are available and the only remedy is strict

avoidance of the allergen [3]. Revealing the underlying mechanisms of sensitization to food proteins may contribute to the development of new treatment methods for peanut allergy.

Lack of oral tolerance due to absence of optimal immunoregulation is thought to be involved in causing food allergy. Preservation of oral tolerance is the result of a complex interplay of a number of cells, including a variety of subsets of regulatory T cells (Tregs). Within the CD4⁺ T cell subpopulation, three classes of Tregs cells

have been distinguished: Th3-type TGF- β -secreting Tregs (Th3), IL-10-producing type 1 Tregs (Tr1), and naturally occurring CD4⁺CD25⁺ Tregs (nTregs) [4]. nTregs were first described by Sakaguchi et al. [5] and are characterized by an anergic phenotype, a constitutive high expression of the IL-2 receptor α chain (CD25), and the expression of the transcription factor forkhead box p3 (FoxP3). Their immunosuppressive activity is most likely mediated by direct cell–cell contact involving for instance CTLA-4 [6] and cell-bound TGF- β [7]. In contrast to nTregs, Tr1 [8, 9] and Th3 [10, 11] cells do not express specific markers and they function through the immunosuppressive cytokines IL-10 and TGF- β , respectively. Additionally, although Tr1 and Th3 cells are activated in an antigen-specific manner, once activated, they can mediate bystander suppressive activity against other antigens, and this bystander suppression is mediated by the local release of IL-10 or TGF- β (Tr1 or Th3 cells, respectively) [12]. The three subsets of Tregs above mentioned have been implicated in the regulation of allergies [13–15].

It has been demonstrated in mouse models that oral exposure to ovalbumin (OVA) induces activation of functional nTregs in gut draining lymph nodes [16] and that adoptive transfers of these activated cells regulate sensitization in OVA TCR-transgenic mice [17]. Studies by van Wijk et al. [13] have shown that depletion of nTregs by anti-CD25 mAb treatment results in an increase of peanut-specific responses, including IgE and mast cell responses. It was also been shown that depletion of nTregs abrogated peanut-specific oral tolerance. It remained unclear, however, if nTregs can transfer allergen-specific oral tolerance to naïve, non-TCR transgenic mice and indeed regulate peanut-specific hypersensitivity responses. To address this issue, CD25-enriched T cells from peanut-tolerant mice were adoptively transferred to recipient mice, which were consequently sensitized to peanut. Our results show that CD25-enriched T cells from orally tolerized mice were unable to transfer tolerance to peanut. However, the presence of CD25⁺ T cells was necessary to control the spontaneous responses transferred within the CD25-depleted population. These results prompted us to further study the role of regulatory cytokines in the modulation of peanut-allergic sensitization. Comparing depletion of CD25 cells with neutralization of IL-10 and TGF- β indicates that both regulatory cytokines are important for general modulation of immune responses, but that TGF- β , hence Th3 T cells are particularly involved in modulating the peanut-specific response.

Materials and methods

Mice

Female, specific pathogen-free C3H/HeOuj Ico mice, 4–5 weeks of age, were purchased from Charles River (Lyon,

France) and were left to acclimatize for 1 week. Mice were maintained under barrier conditions in filter-topped macrolon cages with wood chip bedding, at mean temperature of 23 ± 2 °C, 50–55% relative humidity and 12-h light/dark cycle. Drinking water and standard laboratory food pellets were provided *ad libitum*. The experiments were approved by the animal experiments committee of the Utrecht University.

Chemicals, reagents, and monoclonal antibodies

Anti-CD25 (PC61), anti-IL-10 (JES5.2A5), and anti-TGF- β (1D11) were produced by culturing hybridomas and purified using thiophilic agarose. Cholera toxin (CT) was purchased from List Biological Laboratories Inc. (Campbell, CA, USA). All other chemicals were obtained from Sigma Aldrich (Zwijndrecht, the Netherlands) unless otherwise stated.

Preparation of peanut extract

Peanuts from Golden Peanut (Alpharetta, GA, USA) were kindly donated by Imko Nut Products, the Nut Company (Doetinchem, the Netherlands). Protein extract was made by blending 100 g peanuts with 500 mL of 20 mM Tris buffer (pH 7.2). After 2 h blending at intervals of 20 min for a minute at room temperature, the aqueous fraction was collected by centrifugation (3000 g, at 4 °C for 30 min). The aqueous phase was subsequently centrifuged (10 000 g at 4 °C for 30 min) to remove residual traces of fat and insoluble particles. Protein concentrations were determined using Bradford analysis with BSA as a standard. The extract [peanut extract (PE)] contained typically 32 mg/mL protein and was stored at -20 °C. Reducing SDS-PAGE from the extracts showed protein bands between 14 and approximately 100 kDa (not shown).

Treatment protocols

Oral tolerance induction to PE. Mice ($n = 20$) were exposed by intragastric dosing of 1 mg of PE in 200 μ L PBS for three consecutive days. Control mice ($n = 8$) were exposed to 200 μ L of PBS alone. To determine if tolerance induction was successful and to boost the toleragenic immune response, animals were subsequently sensitized by intraperitoneal (i.p.) challenge on days 21 and 28 with 100 μ g of PE in alum (25 mg/mL). Mice were bled on day 35 and killed on day 43 (Fig. 1a).

Oral sensitization to PE. Mice ($n = 8$) were orally exposed to PBS plus CT (control) or PE plus CT (sensitization). Oral exposure was performed by intragastric dosing of 6 mg of PE plus 10 μ g of CT for three consecutive days, followed

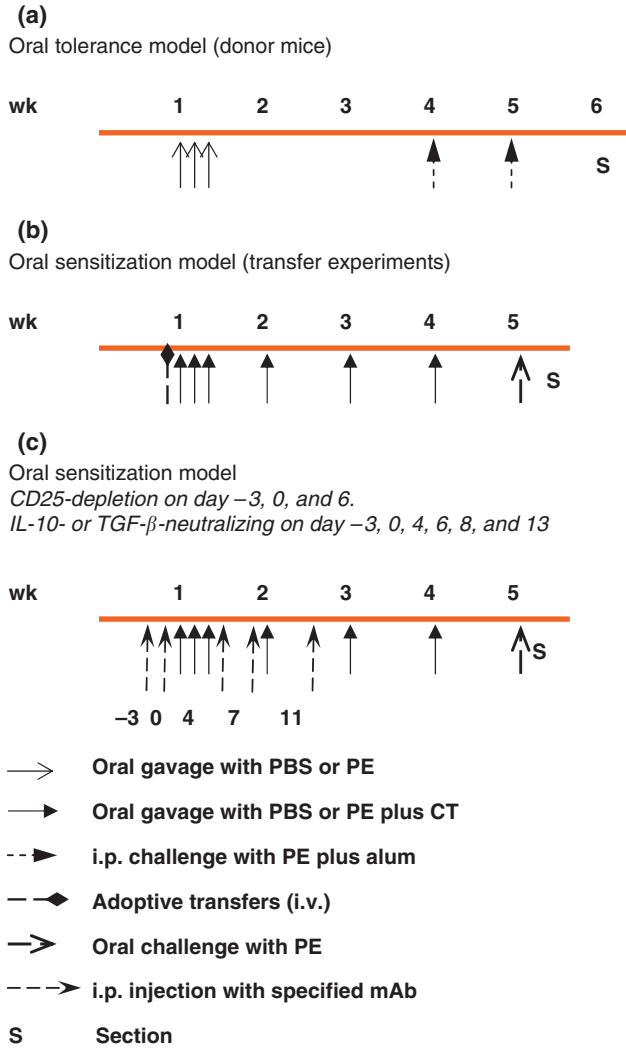


Fig. 1. Schematic overview of treatment protocols. (a) Oral tolerance model (donor mice); (b) oral sensitization model (transfer experiments); (c) oral sensitization model CD25 depletion on day -3, 0, and 6. IL-10- or TGF- β neutralizing on day -3, 0, 4, 6, 8, and 13.

by weekly dosing of PE plus CT (days 7, 14, and 21). At day 28, all groups received an oral challenge with 16 mg of PE and blood was collected 45 min later for measurement of mast cell degranulation. Mice were killed on day 29. For adoptive transfer experiment, 2×10^6 cells were transferred 1 day before initial sensitization to PE (Fig. 1b). For depletion and neutralizing studies, 200 μ g of anti-CD25 mAb (PC61) was given on day -3, 0, and 7, or 500 μ g of anti-IL-10 (JES5.2A5) or anti-TGF- β (1D11) mAbs were given i.p. on days -3, 0, 4, 7, and 11 (Fig. 1c). It has been shown previously that treatment with anti-CD25 monoclonal antibody leads to a drastic decrease in CD4⁺CD25^{high} Tregs [13]. Also, treatment with anti-TGF- β and anti-IL-10 antibodies has been frequently used to show neutralization of their specific cytokines in mice (ref.).

Enrichment of CD4⁺CD25⁺ cells from spleen and mesenteric lymph nodes of tolerized mice

Spleen and mesenteric lymph nodes (MLNs) were isolated from PE-tolerized mice. Single cell suspensions were acquired as described previously and pooled for CD4⁺CD25⁺ enrichment. CD4⁺CD25⁺ enrichment was carried out using a CD4⁺CD25⁺ regulatory T cell isolation kit from Miltenyi Biotec (Bergisch Gladbach, Germany) and was performed according to the manufacturer's instructions.

Measurement of serum IgG1, IgG2a, and IgE antibodies

Serum was collected at various time-points and stored at -20°C until analysis. Levels of PE-specific IgE, IgG1, and IgG2a were determined by ELISA. Plates (highbond 3590; Costar, Cambridge, MA, USA) were coated overnight with $10\text{ }\mu\text{g/mL}$ PE (for IgG1 and IgG2a detection) or with 1.5 mg/mL purified rat anti-mouse IgE (BD Pharmingen, San Diego, CA, USA) in PBS, followed by 1 h blocking with ELISA buffer (50 mM Tris, 136.9 mM NaCl, 2 mM EDTA, 0.5% Tween, and 0.5% BSA). Each test serum was optimally diluted and incubated for 2 h. To determine antibody levels (arbitrary units), a standard curve of the positive reference serum was included. For detection of PE-specific IgG1 and IgG2a, alkaline phosphatase-conjugated IgG1 and IgG2a (both polyclonal goat anti-mouse), 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, a PE-digoxigenin (DIG) conjugate solution (diluted in high-performance ELISA buffer, from Sanquin, San Diego, CA, USA) 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 H_2SO_4 . Absorbance was measured at 450 nm .

Cell culture and cytokine measurement

Spleen and MLN single cell suspensions, 6.25×10^5 cells/mL in RPMI 1640 (Life Technologies, Paisley, Scotland, UK) containing 10% fetal calf serum (ICN Pharmaceuticals, Costa Mesa, CA, USA) were incubated in the presence or absence of 200 $\mu\text{g}/\text{mL}$ 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. The IL-10 (BD Pharmingen) and IFN- γ , IL-4, IL-5, IL-13 ELISA (eBiosciences, San Diego, CA, USA) were

performed in accordance with the manufacturer's instructions.

Measurement of serum mouse mast cell protease-1

On day 28, blood was collected 30–45 min after oral challenge with PE and serum levels of mouse mast cell protease-1 (mMCP-1) were determined using an ELISA kit (Moredun Scientific Ltd, Midlothian, Scotland, UK). ELISA was performed according to the manufacturer's instructions.

Statistics

Data were analysed using GraphPad Prism 4 software. The differences between group means were determined by using one-way ANOVA with Bonferroni as *post hoc* test. Statistical analyses were performed following logarithmic transformation (to achieve normal distribution).

Results

CD25 enrichment captured all FoxP3-expressing cells from spleen and MLN suspensions of tolerized mice

For the transfer studies, CD4⁺CD25⁺ T cells and CD4⁺CD25[−] T cells were isolated from the spleen and MLNs of PE-tolerized donor mice. Oral tolerance was confirmed by lower PE-specific IgE and IgG1 levels in donor mice pretreated with PE than in control mice (Fig. 2a). In addition, splenocytes from donor mice tolerized to PE produced significantly lower amounts of cytokines IL-4, IL-5, IL-10, IL-13, and IFN- γ than those obtained from control mice (Fig. 2b).

CD25 enrichment from the combined suspensions of spleens and MLN from tolerized mice resulted in complete capture of all CD4⁺CD25⁺ FoxP3⁺ Tregs (Fig. 2c). No CD25⁺ or FoxP3⁺ cells were present in the CD25⁺ Tregs-depleted population.

CD25⁺ Tregs cells do not affect PE-specific cytokine production but are rather general by immune suppressive

CD25⁺ Tregs-enriched and -depleted cell populations from PE-tolerized donor mice were transferred to recipient mice 1 day before the standard oral sensitization protocol (with PE + CT) was started (Fig. 1b). After 4 weeks, spleen cells were isolated and cultured in the presence or absence of PE. In agreement with previous studies, significant levels of IL-5, IL-13, and IFN- γ were found upon restimulation with PE in culture supernatants of PE-sensitized mice (Fig. 3). However, these PE-specific T cell responses were not affected by the transfer of CD25⁺ Tregs-enriched or -depleted cells from PE-tolerized donor mice. On the other hand, spontaneous release of all

cytokines was increased in splenocyte cultures of mice that had received CD25⁺ Tregs-depleted cells compared with non-treated sensitized mice and mice that had received CD25⁺ Tregs-enriched cells.

These results show that CD4⁺CD25⁺ FoxP3⁺ Tregs of PE-tolerized mice cannot transfer PE tolerance, but that they are essential for the general suppression of immune responses.

Transfer of CD25⁺ Tregs-enriched populations does not affect PE-specific antibody levels but results in a decrease of mast cell responsiveness

After 4 weeks of exposure to PE plus CT, mice developed PE-specific IgG1, IgG2a, and IgE antibody responses. Adoptive transfers of CD25⁺ Tregs-enriched or -depleted cells had no effect on serum levels of Th1-associated (IgG2a) or Th2-associated (IgG1 and IgE) PE-specific antibody levels compared with non-treated sensitized mice (Fig. 4a).

After the 4-week oral regime, all mice received an oral challenge with PE and the serum mMCP-1 concentrations at 45 min after oral challenge were used as a measure for the mast cell activation and degranulation. Clear mast cell degranulation was observed in six out of eight mice that were sensitized and not treated with cell transfers (Fig. 4b). Although the mean mMCP-1 serum concentration per treated group showed no significant differences to non-treated sensitized mice, the presence of CD25⁺ Tregs-enriched cells in recipient mice resulted in fewer responsive mice (three out of eight are positive) as compared with control groups (six out of eight are positive).

Depletion of CD25 cells or neutralization of TGF- β or IL-10 during PE sensitization results in a profound increase of PE-specific cytokine production

Next to the role of CD4⁺CD25⁺ FoxP3⁺ Tregs, we aimed at investigating the role of regulatory cytokines TGF- β or IL-10, which may also originate from other, e.g. Th3 and/or Tr1, regulatory cells.

Depletion of CD25 Tregs or neutralization of regulatory cytokines TGF- β or IL-10 was carried out during the first 2 weeks of sensitization (Fig. 1c). All antibody treatments resulted in a drastic increase of PE induced as well as spontaneous production of all cytokines measured (Fig. 5). Remarkably, in comparison with anti-CD25 and anti-IL-10 antibody treatment, TGF- β neutralization resulted in a larger increase of spontaneous release of all cytokines measured but interestingly also to a higher PE-specific Th2-associated IL-13 cytokine production. Additionally, IL-10 production was higher in anti-TGF- β treated mice compared with other treatment groups. These data indicate that the regulatory cytokine TGF- β is not only

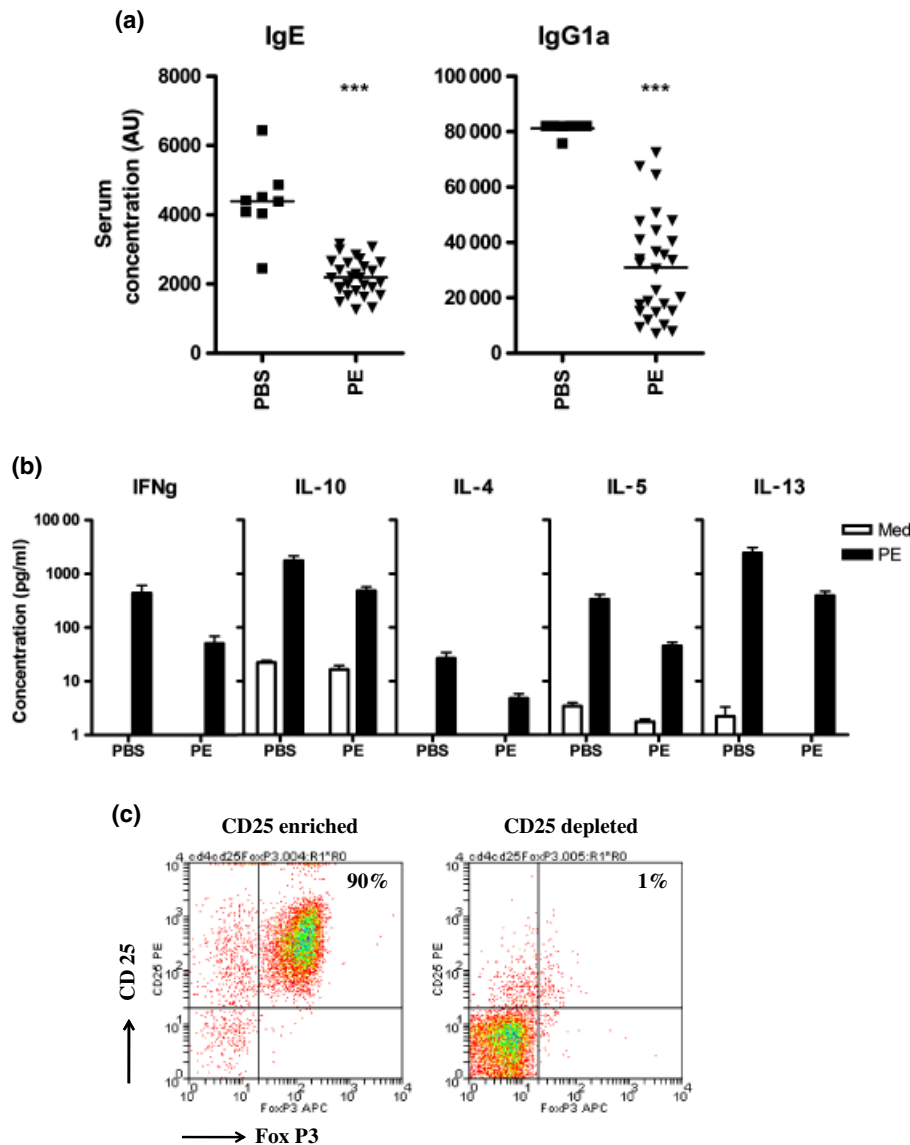


Fig. 2. Characterization of donor spleen and mesenteric lymph nodes cells for adoptive transfers. (a) peanut extract (PE)-specific serum IgE and IgG1 antibodies following PE oral tolerance protocol. Data are represented as the mean serum concentration in arbitrary units of eight or 28 mice per group (symbols indicate individual animals). ***Significantly different ($P < 0.001$) from control PBS pre-treated group. (b) Following the oral tolerance protocol, splenocytes were cultured for 96 h in the presence (black bars) or absence (white bars) of PE. In the supernatant, IL-4, IL-5, IL-10, IL-13, and IFN- γ cytokine levels were determined by ELISA. Data are represented as the group mean of eight mice per group. Restimulation in the presence of peanut lead to a significant difference between PBS and PE pre-treated group for all cytokines ($P < 0.001$). (c) Flow cytometry analysis following enrichment or depletion of CD4⁺CD25⁺ cells. Data are gated on CD4⁺ live cells and %CD25⁺ FoxP3⁺ cells of total CD4⁺ cells injected are indicated.

important for general homeostasis, but particularly involved in modulating Th2 cytokine responses.

Depletion of CD25 cells and TGF- β neutralization but not of IL-10 neutralization result in an increase of PE-specific antibody levels and elevated mMCP-1 release

As anti-CD25, anti-IL-10, and anti-TGF- β all resulted in increased PE-induced cytokine responses, it raised the question whether the humoral response, PE-specific antibody levels, and mMCP-1 levels were also altered.

Treatment with anti-CD25 antibodies significantly increased PE-specific antibody (IgE, IgG1, and IgG2a) production following PE sensitization (Fig. 6a), which confirms previous studies [13]. Treatment with anti-TGF- β showed a slight increase in PE-specific antibodies IgG1 and IgG2a; however, this increase was not observed for PE-specific IgE levels. On the other hand, IL-10 neutralization did not lead to increased PE-specific antibody responses.

In accordance with the increase of PE-specific IgE antibody levels, anti-CD25 treatment also resulted in an increase in mMCP-1 serum level following a PE oral

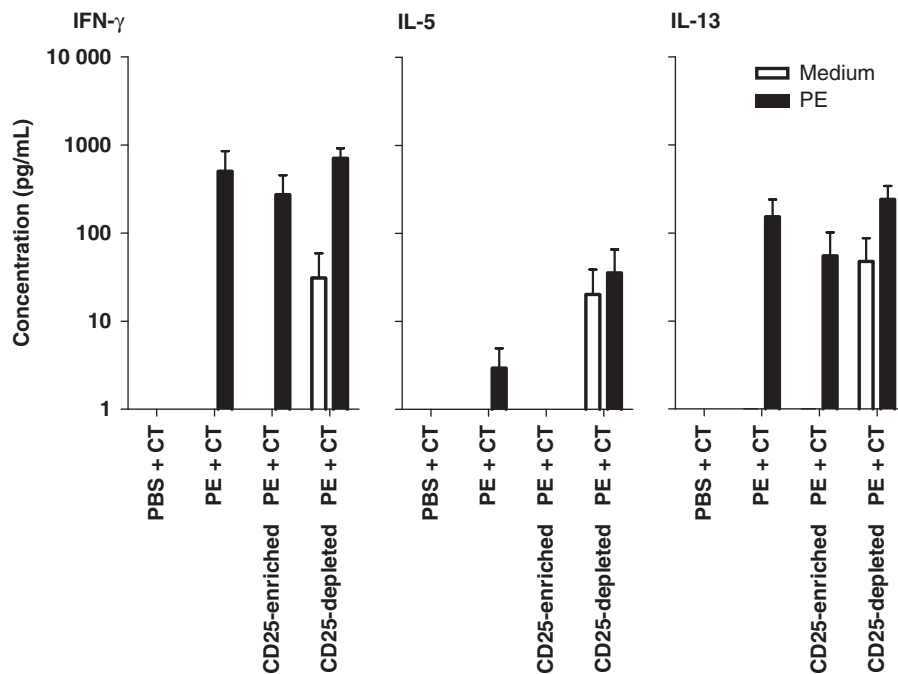


Fig. 3. Effect of CD25-enriched and -depleted cells adoptive transfers on cytokine levels of restimulated splenocytes. Mice were orally sensitized to peanut extract (PE) by exposure to PE+ cholera toxin (CT) or left unsensitized (PBS+CT treatment). Indicated groups received CD25-depleted or -enriched spleen and mesenteric lymph nodes cells 1 day before initial sensitization. Following a 4-week sensitization protocol, splenocytes were cultured for 96 h in the presence (black bars) or absence (white bars) of PE. In the supernatant, IL-5, IL-13, and IFN- γ cytokine levels were determined by ELISA. Data are represented as the group mean of four (unsensitized group) or eight (sensitized groups) mice per group. Groups are significantly different ($P < 0.01$) from the unsensitized group.

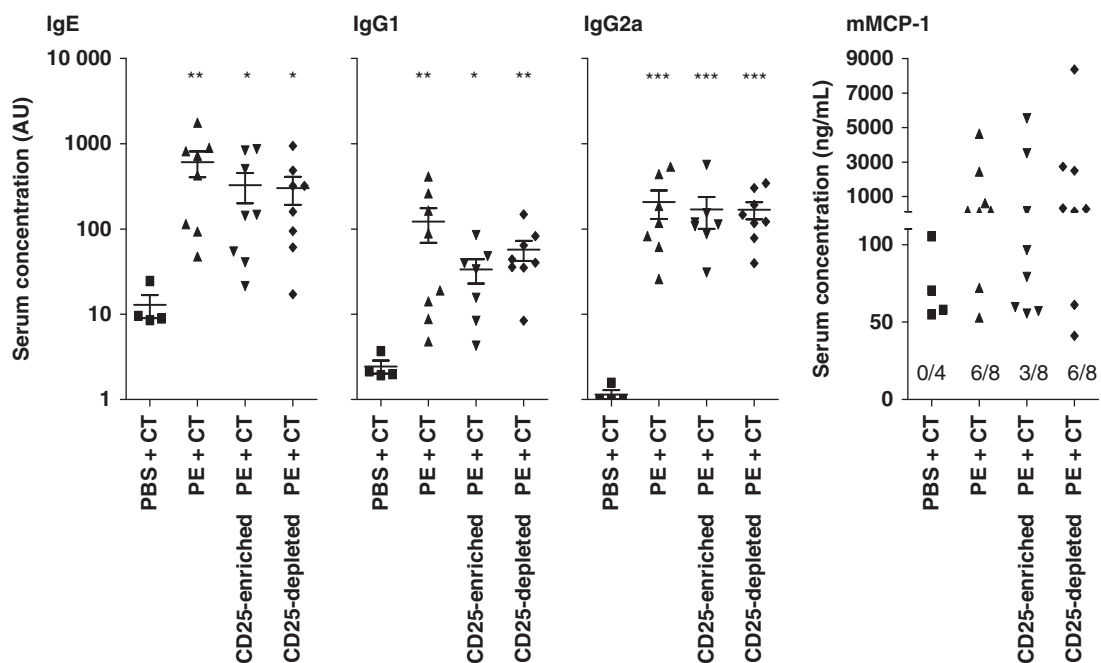


Fig. 4. Effect of CD25-enriched and -depleted spleen and mesenteric lymph nodes cells adoptive transfers on peanut extract (PE)-specific antibody production and mast cell degranulation. Mice were orally sensitized to PE by exposure for 4 weeks to PE+ cholera toxin (CT). One day before the initial sensitization, mice received adoptive transfers of indicated cells. (a) PE-specific serum antibody levels in serum collected after 4 weeks of oral exposure. (b) Mouse mast cell protease-1 (mMCP-1) levels were determined in serum collected within 45 min after an oral challenge with PE. Symbols indicate individual mice and data are presented on a log scale as the mean arbitrary unit per group (a) or in ng/mL (b). *, **, ***Significantly different ($P < 0.05$, $P < 0.01$, $P < 0.001$, respectively) from control unsensitized group. The ratio of responsive mice (mMCP-1 level in serum above highest level in unsensitized group) is stated per group.

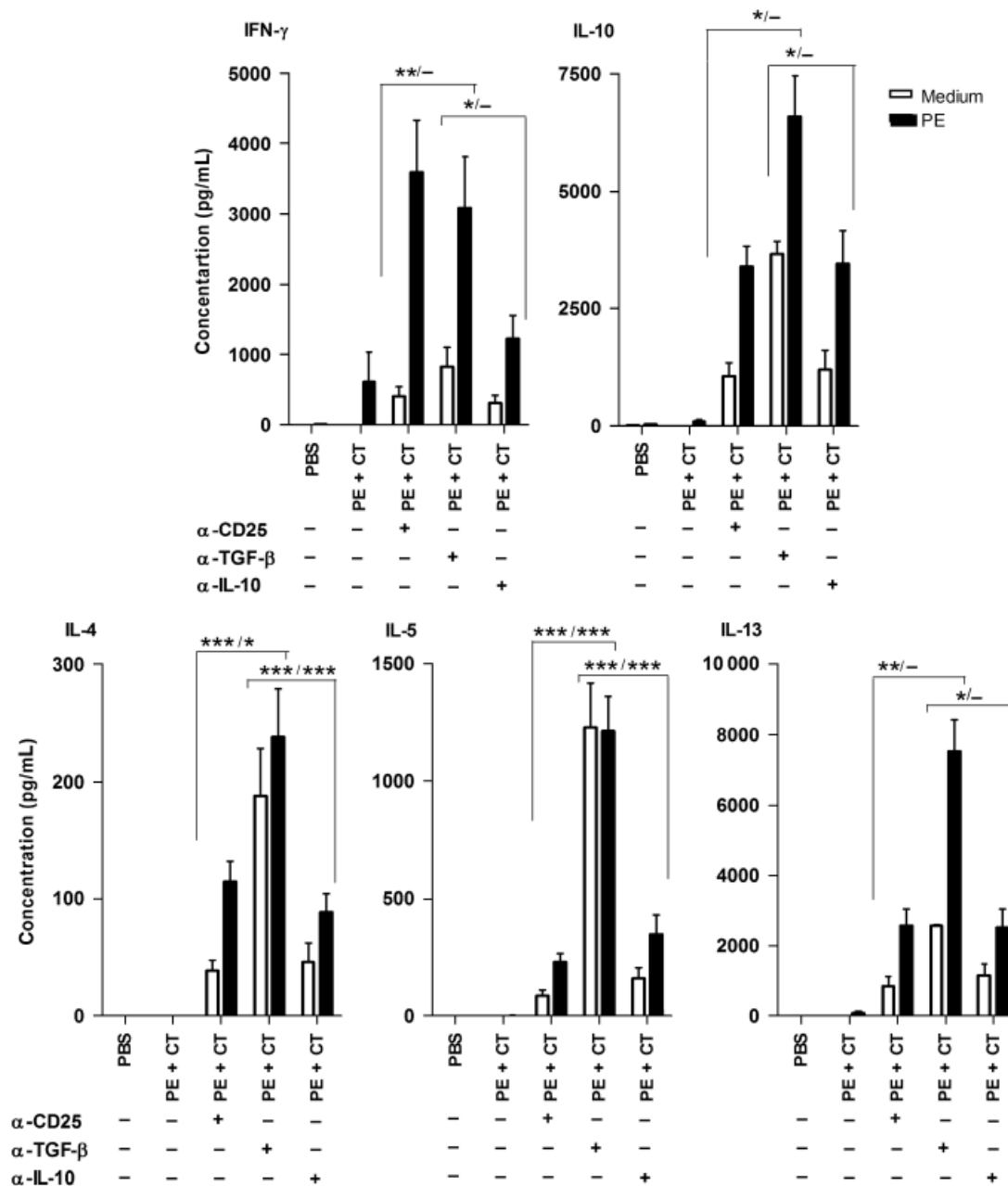


Fig. 5. Effect of CD25 cell depletion or neutralization of TGF- β and IL-10 cytokines on cytokine levels of restimulated splenocytes from peanut extract (PE)-sensitized mice. Mice were orally sensitized to PE by exposure to PE + cholera toxin (CT) or left unsensitized (PBS + CT treatment). Indicated groups received depleting anti-CD25 or neutralizing anti-TGF- β or anti-IL-10 mAbs during the first 2 weeks of PE sensitization. Following a 4-week sensitization protocol, splenocytes were cultured for 96 h in the presence (black bars) or absence (white bars) of PE. In the supernatant, IL-4, IL-5, IL-10, IL-13, and IFN- γ cytokine levels were determined by ELISA. Data are represented as the group mean of five (unsensitized group), six (sensitized control group), and eight (sensitized treated groups) mice per group. *, **, ***Significantly different ($P < 0.05$, $P < 0.01$, $P < 0.001$, respectively) from indicated groups and restimulation condition (medium/PE). All treated groups are significantly different from sensitized mice ($P < 0.05$).

challenge compared with non-treated sensitized mice (Fig. 6b). This increase in mast cell degranulation was also observed following TGF- β neutralization, but not following IL-10 neutralization.

These results show that in contrast to IL-10 neutralization, CD25 depletion and TGF- β neutralization are able to influence both the PE-specific antibody and also mast cell responses.

Discussion

The role of CD4⁺CD25⁺ Tregs in specific modulation of exogenous antigen sensitization in non-transgenic mice is still unclear. Because depletion studies have shown that CD4⁺CD25⁺ Tregs can modulate the sensitization of orally administered PE [13], we investigated whether

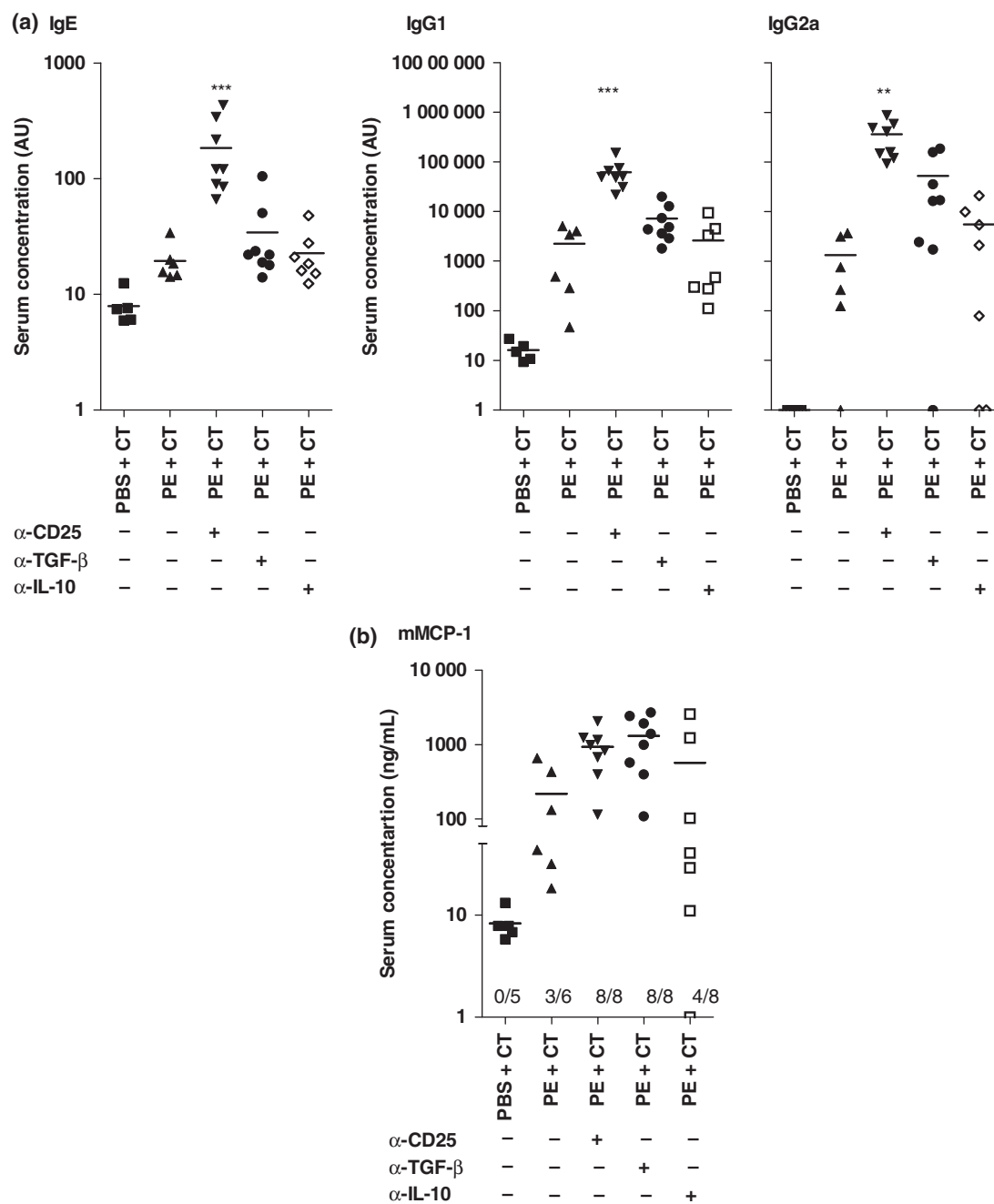


Fig. 6. Effect of CD25 cell depletion or neutralization of TGF- β and IL-10 cytokines on peanut extract (PE)-specific antibody production and mast cell degranulation of PE-sensitized mice. Mice were orally sensitized to PE by exposure to PE + cholera toxin (CT) or left unsensitized (PBS + CT treatment). Indicated groups received depleting anti-CD25 or neutralizing anti-TGF- β or anti-IL-10 mAbs during the first 2 weeks of PE sensitization. (a) PE-specific antibody levels in serum collected after 4 weeks of oral exposure. (b) Mouse mast cell protease-1 (mMCP-1) levels were determined in serum collected within 45 min after an oral challenge with PE. Symbols indicate individual mice. Data were logarithmically transformed and are presented linear as the mean arbitrary unit per group (a) or in ng/mL (b). **, *** Significantly different ($P < 0.01$ and $P < 0.001$, respectively) from the sensitized group. The ratio of responsive mice (mMCP-1 level in serum above highest level in unsensitized group) is stated per group.

transfer of isolated CD4⁺CD25⁺ Tregs from tolerized mice could prevent oral sensitization to PE. Our results show that CD4⁺CD25⁺ Tregs are unable to transfer PE-specific oral tolerance to naïve mice but that they are essential for homeostasis, because transfer of CD25⁺ Tregs-depleted

cells showed an increase in non-specific cytokine production. This is in agreement with the present and previous depletion studies, showing an increase in both non-specific and PE-specific cytokine responses when CD25⁺ cells are depleted [13]. However, depletion of CD25⁺ cells

also resulted in increases of serum levels of PE-specific antibodies and mMCP-1, while this effect was not observed when CD25-depleted cells, were transferred. We have used the current approach to investigate whether transfer of Tregs could down-regulate orally induced food allergy responses. However, PE+CT treatment itself also induces these (and other) regulatory cells [13] and they may interfere with the efficacy of transferred cells. Nonetheless, with the transfer of CD25-depleted cells we observed a clear change in spontaneous cytokine production. It is important to note in this respect that adoptive transfer of Tregs is a much more gentle manipulation of the system than depletion of cell populations or neutralization of cytokines, which may have a larger impact on homeostasis. This may be an explanation as to why Treg cell depletion in the peanut sensitization and oral tolerance model shows such drastic differences in responses in comparison with adoptive transfers.

As Sakaguchi et al. [5] first described CD4⁺CD25⁺ Tregs, a number of studies have meticulously studied their function and suppressive properties, both *in vitro* and *in vivo*. They have shown the importance of CD4⁺CD25⁺ Tregs in preventing the development of autoimmune diseases [5, 18–20] and in effective tumour immunity [21–23]. Adoptive transfer studies have shown a role of CD4⁺CD25⁺ Tregs in the modulation of auto-immune diseases [5, 18, 20]. With respect to non-auto-antigens, it has been shown that oral administration of OVA to OVA TCR-transgenic (DO11.10) mice can induce CD4⁺CD25⁺ Tregs that can transfer specific-tolerance to wild-type mice [17]. However, TCR-transgenic mice of the DO11.10 strain, harbouring a majority of T cells that recognizes only a single antigen, do not reflect a normal situation. One could speculate that nTregs are necessary for the general homeostasis and auto-tolerance in the periphery, while other suppressive immune cells or factors may be necessary to establish and maintain oral tolerance in the gut. The immunosuppressive environment of the gut has been reviewed by many in the last years [24, 25], and the regulatory cytokines IL-10 and TGF- β have frequently been suggested as important regulatory cytokines. CD4⁺CD25⁺ Tregs are believed to function through cell-cell contact but they may still modulate sensitization to exogenous antigens by bystander cytokine-mediated suppression [12]. Similarly, both Tr1 and Th3 Tregs may be responsible in bystander suppression. In all cases, the

regulatory cytokines IL-10 and TGF- β may be very important.

Previous studies show that TGF- β -secreting Th3 cells mainly inhibit Th1 responses, while regulation of Th2 responses, including IgE responses to oral allergens has been particularly attributed to IL-10-producing Tr1 cells [26, 27]. Nevertheless, peanut allergy in the present mouse model implicates both Th1 and Th2 immune responses [28], permitting a possible role for both Tr1 and Th3 cells for the modulation of allergic sensitization to PE. However, Tr1 cells have been described to express CD25 in response to antigen activation [29], which means their presence within the CD25⁺ Tregs-enriched population cannot be excluded. Tr1 cells have furthermore been frequently implicated in tolerance in the periphery, while Th3 cells have been extensively described in the gut [10, 11]. In the present study, results of neutralization of IL-10 suggest that IL-10 does not have a strong regulatory effect. In another study, we observed that by using more extended dosing of neutralizing antibodies during all 4 weeks of PE sensitization, IL-10 is very well capable of regulating Th1/Th2 cytokine production and mast cell responses. However, data suggest that IL-10 is less efficient in modulating food allergy responses than for instance IFN- γ and TGF- β (data not shown).

Th3 cells constitute a unique T cell subset, which primarily secretes TGF- β . Our neutralization studies show that TGF- β serves as an important regulatory cytokine during oral PE sensitization because TGF- β neutralization has to significantly more cytokine production by restimulated splenocytes than neutralization of IL-10 or depletion of CD25⁺ cells, and to an increase in PE-specific antibodies and mast cell degranulation. This is in line with other studies, which have shown that immune responses to soluble antigens in the gut involve the generation of cells that secrete TGF- β (Th3 cells) [30]. Furthermore, TGF- β is a pleiotropic cytokine that contributes to the maintenance of the intestinal epithelial barrier and to the reduction of pro-inflammatory stimuli [31].

We conclude that CD4⁺CD25⁺ Tregs from non-transgenic-tolerant mice do not transfer specific oral tolerance of exogenous antigens to naïve mice under the used conditions. Even so, much more research is needed the role of Tregs, in particular of the Th3 cells, in the modulation of allergic sensitization and the maintenance of oral tolerance.

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