

Regulatory activity of human CD4⁺ CD25⁺ T cells depends on allergen concentration, type of allergen and atopy status of the donor

Iris Bellinghausen, Bettina König,
Ingo Böttcher, Jürgen Knop and
Joachim Saloga

Department of Dermatology, University of
Mainz, Mainz, Germany

Summary

Regulatory CD4⁺ CD25⁺ FoxP3-positive T cells (Treg) are functional in most atopic patients with allergic rhinitis and are able to inhibit T helper type 1 (Th1) and Th2 cytokine production of CD4⁺ CD25⁻ T cells. This study was designed to analyse the following additional aspects: influence of allergen concentration, influence of the type of allergen, and influence of the atopy status of the donor on the strength of the regulatory activity. CD4⁺ CD25⁻ T cells from healthy non-atopic controls or from grass-pollen-allergic or wasp-venom-allergic donors were stimulated alone or in the presence of Treg with autologous mature monocyte-derived dendritic cells which were pulsed with different concentrations of the respective allergens. Treg from grass-pollen-allergic donors failed to inhibit proliferation but not cytokine production of CD4⁺ CD25⁻ T cells at high antigen doses while Treg from non-atopic donors did not fail at these allergen concentrations. Proliferative responses and cytokine production of CD4⁺ CD25⁻ T cells from most of the examined wasp-venom-allergic patients were not inhibited at any concentration of wasp venom. The use of wasp venom- or phospholipase A₂-pulsed dendritic cells for stimulation of CD4⁺ CD25⁻ T cells from donors who were not allergic to wasp stings only resulted in an inhibited proliferation and Th2 cytokine production by Treg at 10-fold lower than the optimal concentration, while interferon- γ production was inhibited at all concentrations investigated. These data demonstrate that in allergic diseases the function of Treg is dependent on the concentration and the type of the respective allergen with different thresholds for individual allergens and patients.

Keywords: allergy; CD25; dendritic cells; human regulatory T cells (Treg); Th1/Th2

doi:10.1111/j.1365-2567.2005.02205.x

Received 30 November 2004; revised 5 April 2005; accepted 4 May 2005.

Correspondence: Dr Iris Bellinghausen,
Universitäts-Hautklinik, Langenbeckstraße 1,
D-55131 Mainz, Germany. Email:
bellinghausen@hautklinik.klinik.uni-mainz.de
Senior author: Prof. Joachim Saloga,
email: saloga@hautklinik.klinik.uni-mainz.de

Introduction

Peripheral tolerance can be achieved by different suppressor T-cell populations. T regulatory 1 (Tr1) cells are induced mainly in the presence of interleukin-10 (IL-10), produce high levels of IL-10 and transforming growth factor- β (TGF- β), and suppress activation and cytokine production of effector T cells in a cell contact-independent but IL-10-dependent manner. T helper type 3 (Th3) cells produce TGF- β , IL-10 and IL-4, and suppress via a

TGF- β -dependent mechanism. CD4⁺ CD25⁺ T regulatory cells (Treg) inhibit IL-2 production in CD4⁺ and CD8⁺ effector T cells which requires cell to cell contact and is not mediated by IL-10, TGF- β or cytotoxic T-lymphocyte antigen-4 (CTLA-4).^{1–3} Treg were originally described in mice, where they are responsible for protection against various autoimmune diseases, such as gastritis or thyroiditis.^{4,5} Unlike Tr1 cells, they arise in the thymus and represent a stably anergic cell lineage. Furthermore, they are characterized by the expression of CTLA-4 and the

Abbreviations: CAP, capacity of binding by the Immuno CAP[®] Specific IgE blood test; DC, dendritic cells; DTAF, dichlorotriazinylamino-fluorescein; Tr1 cells, T regulatory 1 cells; Treg, regulatory T cells.

glucocorticoid-induced tumour necrosis factor receptor family related gene (GITR) which play an essential role in their regulatory function.^{1,6–10} One recently defined more specific marker for Treg in mice seems to be the forkhead transcription factor FoxP3, as it has been shown that the lethal autoimmune syndrome observed in FoxP3-mutant scurfy mice results from a deficiency in Treg.^{11,12}

In humans, 2–10% of all peripheral CD4⁺ T cells are referred to as CD4⁺ CD25⁺ Treg. They do not proliferate after allogeneic or polyclonal activation and once activated, they suppress the proliferation and cytokine production of CD4⁺ and CD8⁺ T cells in an antigen-non-specific, cytokine-independent, but cell-contact-dependent, manner.^{13–15} Like their murine counterparts, expression of CTLA-4, GITR, and FoxP3 is not exclusively found in CD4⁺ CD25⁺ Treg but is also found in activated CD4⁺ CD25[−] T cells.^{16,17} Recently, we and others have investigated the role of CD4⁺ CD25⁺ Treg in allergic diseases.^{3,18–21} We have shown that most of the examined atopic or allergic patients also possess CD4⁺ CD25⁺ FoxP3-positive Treg with intact regulatory functions.¹⁸ In the present study we demonstrate that the suppressive activity of CD4⁺ CD25⁺ Treg is dependent on the allergen concentration and type of allergen with different thresholds for different allergens and individuals correlated with their sensitization/atopy status.

Materials and methods

Patients

Heparinized blood was obtained from healthy non-atopic controls or from atopic donors with allergic rhinoconjunctivitis (some suffered additionally from asthma) to grass pollen or donors with severe systemic reactions to wasp stings (i.e. generalized urticaria, angioedema, hypotension, or shock). Specific sensitization was documented by positive skin prick test to the respective allergen and detection of allergen-specific immunoglobulin E (IgE) in the sera [capacity of binding by the Immuno CAP[®] Specific IgE blood test (CAP: Pharmacia Deutschland GmbH, Freiburg, Germany)-class ≥ 2]. Two of the examined seven wasp-allergic patients were also sensitized to grass pollen.

Blood samples were taken both during and outside the pollen season. This study was approved by the local ethics committee. Informed consent was obtained from all subjects before the study.

Cell culture reagents and allergens

Iscove's modified Dulbecco's medium with L-glutamine and 25 mM HEPES (IMDM, Life Technologies GmbH, Karlsruhe, Germany) supplemented with 3024 mg/l sodium bicarbonate, 100 µg/ml streptomycin, 100 U/ml penicillin, and 1% heat-inactivated autologous plasma

was used for the culture of dendritic cells (DC) and with 5% heat-inactivated autologous plasma or AB-serum from non-allergic individuals (Sigma, Taufkirchen, Germany) for the coculture of T cells and DC.

Human recombinant IL-4, IL-1 β and tumour necrosis factor- α (TNF- α) were purchased from Strathmann Biotech GmbH (Hannover, Germany), granulocyte-macrophage colony-stimulating factor (GM-CSF; Leucomax[®]) was obtained from Sandoz AG (Nürnberg, Germany) and prostaglandin E₂ (Minprostin[®]) was supplied by Pfizer GmbH (Karlsruhe, Germany). The grass pollen and wasp venom allergen extracts were provided by ALK-Schering (Hamburg, Germany). Phospholipase A₂ from honey bee was provided by Sigma. The endotoxin content of all allergen extracts was tested with QCL-1000 chromogenic LAL (Bio-Whittaker, Walkersville, MD) according to the instructions of the manufacturer, and only batches with a contamination of endotoxin < 1 ng/ml were used.

Antibodies

The following monoclonal antibodies were used. Mouse IgG was anti-CD80 (MAB104, Immunotech, Hamburg, Germany), anti-CD83 (HB15a, Immunotech), and anti-CD86 (BU63, Camon, Wiesbaden, Germany). Rat IgG was anti-HLA-DR (YE2/36HLK, Camon), polyclonal goat IgG was anti-GITR (N-14, Santa Cruz Biotechnology Inc., Santa Cruz, CA), and mouse, rat and goat subclass-specific isotypes (Immunotech or Santa Cruz Biotechnology Inc.). Conjugated secondary antibodies were fluorescein (dichlorotriazinylamino-fluorescein; DTAF)-conjugated goat anti-rat IgG, phycoerythrin (PE)-conjugated donkey anti-mouse IgG, and PE-conjugated donkey anti-goat IgG (all from Dianova, Hamburg, Germany). Fluorescein isothiocyanate- (FITC) or PE-conjugated antibodies for staining of magnetic antibody cell-sorted T cells: anti-CD4 (RPA-T4), anti-CD25 (M-A251), and FITC- and PE-conjugated mouse or rat IgG isotype controls (Immunotech).

Generation of monocyte-derived DC

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood by Ficoll-Paque 1.077 (Biochrom, Berlin, Germany) density centrifugation. To enrich CD14⁺ monocytes 1×10^7 PBMC per well were incubated for 45 min in a six-well-plate (Costar, Bodenheim, Germany) in IMDM supplemented with 3% autologous plasma at 37°. After washing the non-adherent cells with prewarmed phosphate-buffered saline the remaining monocytes (purity > 90%) were incubated in 3 ml/well IMDM supplemented with 1% heat-inactivated autologous plasma, 1000 U/ml IL-4 and 800 U/ml GM-CSF. Cells were fed with fresh medium every other day. On day 7 the resulting immature DC were pulsed with, unless otherwise

indicated, 5 µg/ml grass pollen, or 1 µg/ml wasp venom allergen extracts, and further stimulated with 1000 U/ml TNF- α , 2000 U/ml IL-1 β and 1 µg/ml prostaglandin E₂ to induce their full maturation. After 48 hr, mature DC were harvested, washed twice and used for T-cell stimulation assays. Mature DC expressed high levels (> 90%) of CD80, CD83, CD86 and major histocompatibility complex-class II molecules as controlled by flow cytometry.

Purification of T cells

Autologous CD4⁺ T cells were obtained from PBMC using antibody-coated paramagnetic MultiSort MicroBeads (MACS, Miltenyi Biotec, Bergisch Gladbach, Germany) according to the protocol of the manufacturer. After detaching, CD4⁺ T cells were stained with CD25 MicroBeads (Miltenyi Biotec) and CD4⁺ CD25⁺ T cells were positively selected and CD4⁺ CD25⁻ T cells were negatively selected. Separation was controlled by flow cytometry (purity >98% CD4⁺ T cells, >98% CD4⁺ CD25⁻ T cells, and >95% CD4⁺ CD25⁺ T cells).

Coculture of T cells and autologous allergen-pulsed DC

For proliferation assays, 1×10^5 CD4⁺ CD25⁻ T cells, 1×10^5 CD4⁺ CD25⁻ T cells plus 5×10^4 CD4⁺ CD25⁺ Treg, or 1×10^5 CD4⁺ CD25⁺ Treg were cocultured in 96-well-plates (Costar) in triplicate with 1×10^4 autologous allergen-pulsed DC in 200 µl IMDM supplemented with 5% heat-inactivated autologous plasma or AB-serum. After 5 days, the cells were pulsed with 37 kBq/well of [³H]TdR (ICN, Irvine, CA) for 6 hr, and [³H]TdR incorporation was evaluated in a beta-counter (1205 Beta-plate, LKB Wallac, Turku, Finland).

For cytokine production assays, 5×10^5 CD4⁺ CD25⁻ T cells, 5×10^5 CD4⁺ CD25⁻ T cells plus 2.5×10^5 CD4⁺ CD25⁺ Treg, or 5×10^5 CD4⁺ CD25⁺ Treg were cocultured in 48-well-plates in the presence of 5×10^4 autologous allergen-pulsed DC in 1 ml IMDM supplemented with 5% heat-inactivated autologous plasma or AB-serum. On day 7, T cells were restimulated with 5×10^4 newly generated autologous allergen-pulsed DC, and supernatants were collected 24 hr later.

Flow cytometric analysis

Surface phenotyping was performed by staining 5×10^5 T cells or 5×10^4 DC with specific monoclonal antibodies for 20 min at 4°. After being washed with phosphate-buffered saline containing 0.1% bovine serum albumin, the cells were incubated with DTAF- or PE-conjugated secondary antibodies for 20 min at 4°, washed, and analysed in a FACSCalibur (Becton Dickinson, Mountain View, CA) equipped with CELLQUEST Software.

Quantification of cytokine production by ELISA

Human IL-4, IL-10 and interferon- γ (IFN- γ) were measured by ELISA according to the instructions of the distributors of the employed pairs of antibodies (BD PharMingen). The detection limit was 8 pg/ml for IL-4 and 32 pg/ml for IL-10 and IFN- γ .

Determination of RNA expression for FoxP3 by reverse transcription-polymerase chain reaction

CD4⁺ CD25⁻ or CD4⁺ CD25⁺ T cells were prepared as described above and total RNA was extracted using the RNeasy Mini Kit (QIAGEN GmbH, Hilden, Germany) following the instructions of the manufacturer. Then, 100 ng of RNA was reverse transcribed in a 20-µl reaction containing oligo-dT primers and Omniscript[®] reverse transcriptase (QIAGEN), and the cDNA was amplified by 30 cycles using the HotStarTaq[®] DNA Polymerase Master Mix Kit (QIAGEN). The cycling conditions chosen were 15 min at 96°; 30 cycles of 1 min at 94° (denaturation), 1 min at 55° (annealing) and 30 seconds at 72° (extension); and 10 min final extension. The primer sequences for Foxp3 and β -actin were as follows: FoxP3 forward, 5'-CTA CGC CAC GCT CAT CCG CTG G-3'; FoxP3 reverse, 5'-GTA GGG TTG GAA CAC CTG CTG GG-3'; β -actin forward, 5'-GGA CTT CGA GCA AGA GAT GG-3'; β -actin reverse, 5'-AGC ACT GTG TTG GCG TAC AG-3'. In each experiment, amplifications were performed without addition of RNA and primers as a negative control. The polymerase chain reaction products were separated for 1 hr by agarose gel electrophoresis (2%) and stained with ethidium bromide. For analysing the molecular size of the polymerase chain reaction amplicates a 100-base-pair DNA ladder (MBI Fermentas GmbH, St Leon-Rot, Germany) was used as a standard.

Statistical analysis

Student's *t*-test was employed to test the statistical significance of the results; $P \leq 0.05$ was considered significant.

Results

Freshly isolated CD4⁺ CD25⁺ Treg from allergic and non-atopic donors express GITR and FoxP3

First, we characterized the freshly isolated CD4⁺ CD25⁺ Treg concerning their expression of the recently defined marker GITR and FoxP3. In allergic and in non-atopic donors alike CD4⁺ CD25⁺ Treg were almost all positive for GITR compared to CD4⁺ CD25⁻ peripheral T cells ($91.3 \pm 3.7\%$ of CD4⁺ CD25⁺ Treg versus $14.8 \pm 5.7\%$ of

CD4⁺ CD25⁻ T cells, $n = 10$). Additionally, CD4⁺ CD25⁺ Treg from allergic as well as non-atopic donors showed a strong signal for FoxP3 expression while CD4⁺ CD25⁻ T cells did not.

CD4⁺ CD25⁺ Treg from grass-pollen-allergic patients fail to inhibit proliferation but not cytokine production of CD4⁺ CD25⁻ responder T cells at high allergen doses while Treg from non-atopic donors retain their regulatory properties

As it has been demonstrated that increased costimulation or maximal anti-CD3 stimulation breaks CD4⁺ CD25⁺ Treg-mediated suppression,^{22,23} we investigated whether the regulatory capacity of CD4⁺ CD25⁺ Treg may also be overcome using higher concentrations of the respective allergen in our coculture system. Therefore, CD4⁺ CD25⁻ responder T cells from grass-pollen-allergic and non-atopic donors were stimulated in the presence or absence of CD4⁺ CD25⁺ Treg with autologous mature DC pulsed with from 0.4 up to 50 µg/ml grass pollen allergen. Using high allergen doses (50 µg/ml) proliferation of CD4⁺ CD25⁻ T cells from grass-pollen-allergic patients was not suppressed by CD4⁺ CD25⁺ Treg showing themselves a high proliferative response at this allergen concentration in contrast to the proliferation of CD4⁺ CD25⁻ T cells from non-atopic donors which was suppressed by CD4⁺ CD25⁺ Treg at all concentrations tested (Fig. 1a). Th1 and Th2 cytokine production of CD4⁺ CD25⁻ T cells from both groups of donors were inhibited by CD4⁺ CD25⁺ Treg at all allergen concentrations and the production of IL-10 was not affected by Treg at any concentration (Fig. 1b). Except for the marked proliferation of Treg from grass-pollen-allergic patients at the highest allergen concentration, Treg from non-atopic donors or from grass-pollen-allergic patients showed no, or only a little, proliferation and cytokine production (Fig. 1a,b). The use of CD4⁺ CD25⁺ Treg at a ratio of 1 : 1 did not prevent failure of suppression at high allergen concentration in allergic donors (data not shown). In addition, the use of AB serum from non-allergic donors, which did not contain allergen-specific IgE, did not change the results except for generally a slightly reduced proliferative response in all conditions (data not shown).

CD4⁺ CD25⁺ Treg from wasp-venom-allergic patients fail to inhibit proliferation and Th2 cytokine production even at low allergen concentrations

For the following experiments we pulsed DC during their maturation phase with a concentration of wasp venom (1 µg/ml) resulting in an optimal proliferation index of CD4⁺ T cells in our coculture system which has been investigated by us in prior studies and with 10- and 100-

fold lower concentrations. At each concentration of wasp venom used for a DC pulse, CD4⁺ CD25⁺ Treg from wasp-venom-allergic patients showed almost the same or even higher proliferative responses and Th2 cytokine and IL-10 production compared to CD4⁺ CD25⁻ T cells and consequently we did not observe any suppression. The production of the Th1 cytokine IFN-γ by CD4⁺ CD25⁺ Treg was lower than that of CD4⁺ CD25⁻ T cells with a slight but not significant suppressive activity of CD4⁺ CD25⁺ Treg by this cytokine (Fig. 2a,b). In some patients, the lack of regulation concerning proliferation and Th2 cytokine production was wasp venom-specific as regulation could be observed using grass-pollen-pulsed DC as antigen presenting cell. However, there were also patients whose Treg showed no regulatory function after stimulation with grass-pollen-pulsed DC (data not shown).

In the context of a strong allergen like wasp venom or phospholipase A₂ CD4⁺ CD25⁺ Treg, even from donors who are not allergic to wasp venom, inhibit proliferation and Th2 cytokine production only at low (suboptimal) allergen doses

Next, we analysed the regulation of CD4⁺ CD25⁻ responder T cells by CD4⁺ CD25⁺ Treg from donors who were not sensitized to wasp venom using DC pulsed with 1 µg/ml wasp venom for stimulation (optimal concentration). Under these conditions CD4⁺ CD25⁺ Treg proliferated normally without any suppressive activity, as shown in Fig. 3(a). In contrast, stimulation of the same cells with DC pulsed with an optimal dose of grass pollen allergen (5 µg/ml) resulted in a suppressed proliferation of CD4⁺ CD25⁻ T cells by CD4⁺ CD25⁺ Treg. Th2 cytokine production was also not inhibited by CD4⁺ CD25⁺ Treg stimulated with wasp-venom-pulsed DC compared to grass-pollen-allergen-pulsed DC while the production of IFN-γ was reduced in both stimulation models. Again, the production of IL-10 was not significantly inhibited by Treg in cocultures (using wasp-venom-pulsed DC it was even rather enhanced probably as a result of the IL-10 production of Treg themselves) (Fig. 3b).

To investigate whether the regulatory activity of CD4⁺ CD25⁺ Treg from donors who were not allergic to wasp venom was retained at 10-fold lower allergen concentrations, DC pulsed with 0.1 µg/ml wasp venom were used for stimulation in cocultures with CD4⁺ CD25⁻ T cells. In these cocultures, proliferative responses and cytokine production of CD4⁺ CD25⁺ Treg were low and proliferation as well as Th1 and Th2 cytokine production of CD4⁺ CD25⁻ T cells were suppressed (Fig. 3a,b).

Finally, we analysed whether the loss of regulatory functions by CD4⁺ CD25⁺ Treg at high wasp venom concentrations was the result of the major allergen phospholipase

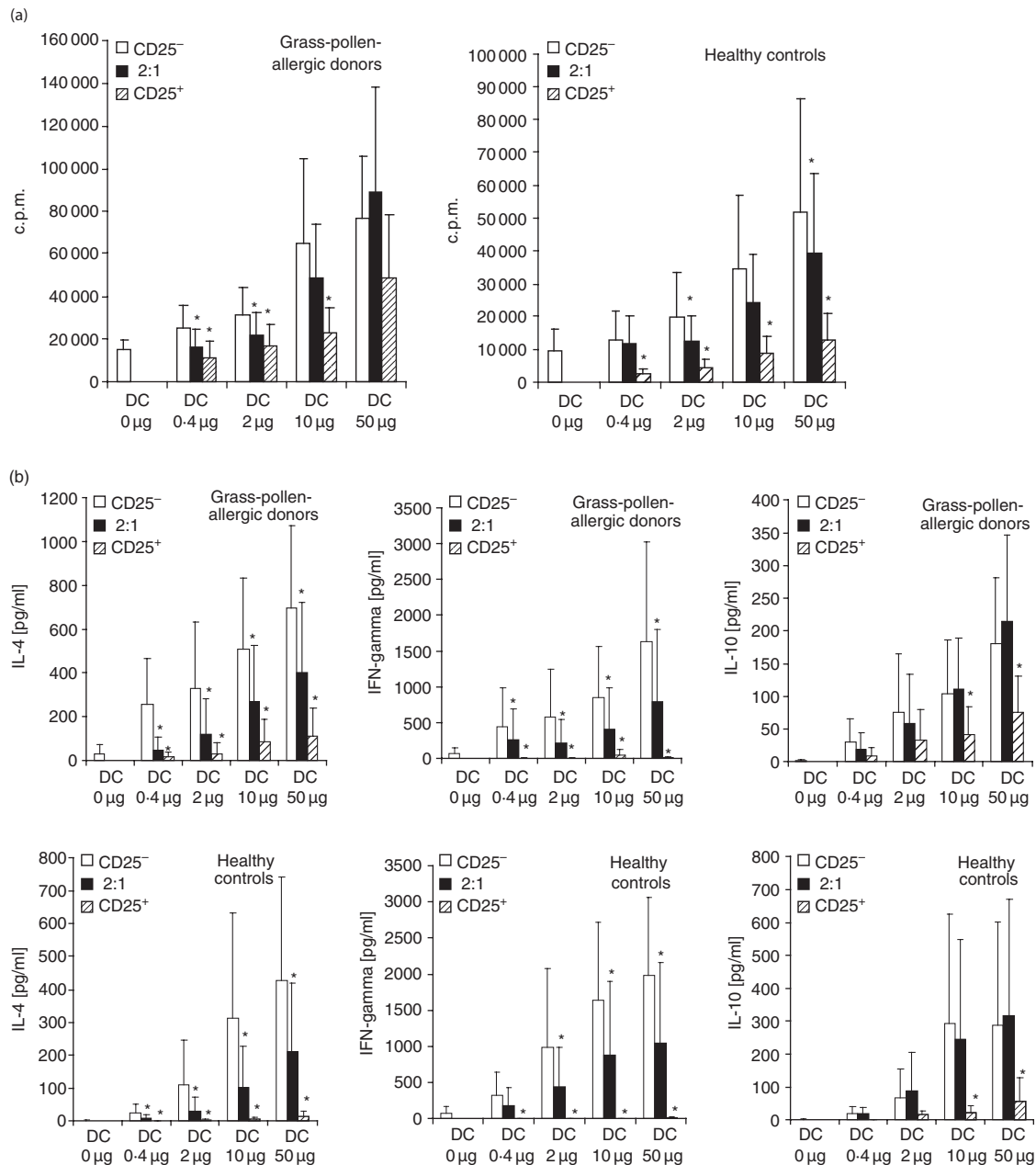


Figure 1. Treg-mediated suppression of proliferation of CD4⁺ CD25⁻ T cells from grass-pollen-allergic patients but not from healthy donors is impaired at high allergen doses. CD4⁺ CD25⁻ T cells (CD25⁻) cultured alone or in the presence of CD4⁺ CD25⁺ Treg (2 : 1) or Treg alone (CD25⁺) were stimulated with DC pulsed with the indicated concentrations of grass pollen allergen. (a) For analysis of proliferation [³H]TdR incorporation was measured at day 5. (b) For cytokine analysis the cocultures were restimulated with newly generated allergen-pulsed DC on day 7. Supernatants were collected 24 hr later and analysed for IL-4, IL-10, and IFN- γ content by ELISA. The means $\pm$ SD from 10 grass-pollen-allergic and 12 healthy donors are shown. *Represents statistically significant differences between CD4⁺ CD25⁻ T cells alone versus coculture with Treg or Treg alone, $P \leq 0.05$.

A₂. In Fig. 3(c) we demonstrate that the use of an optimal dose of phospholipase A₂ also led to a failure in CD4⁺ CD25⁺ Treg-mediated suppression of CD4⁺ CD25⁻ T-cell proliferation while at 10-fold lower concentrations suppression was retained.

Discussion

The development of allergic diseases such as rhinitis, asthma and atopic eczema is controlled by several populations of regulatory T cells including CD4⁺ CD25⁺ Treg.²⁴

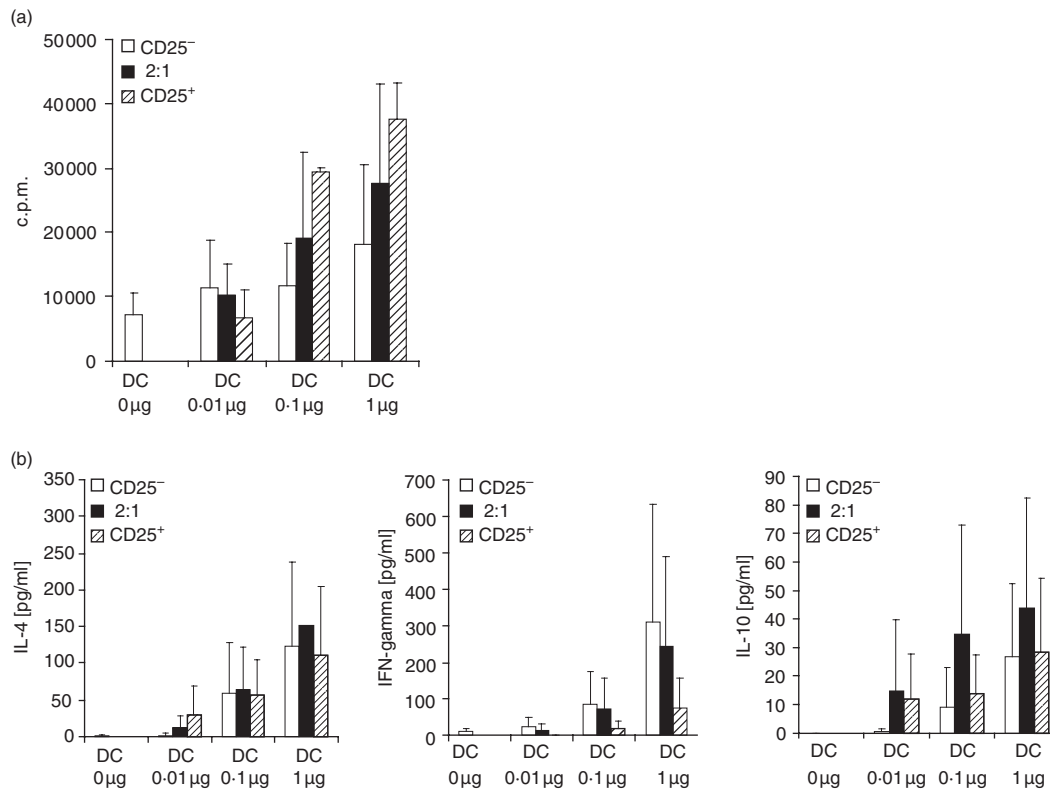


Figure 2. CD4⁺ CD25⁺ Treg-mediated suppression of proliferation and Th2 cytokine production of CD4⁺ CD25⁻ T cells from wasp-venom-allergic patients is impaired at any tested allergen concentration. CD4⁺ CD25⁻ T cells (CD25⁻) cultured alone or in the presence of CD4⁺ CD25⁺ Treg (2 : 1) or Treg alone (CD25⁺) were stimulated with DC pulsed with the indicated concentrations of wasp venom. (a) For analysis of proliferation [³H]TdR incorporation was measured at day 5. (b) For cytokine analysis the cocultures were restimulated with newly generated allergen-pulsed DC on day 7. Supernatants were collected 24 hr later and analysed for IL-4, IL-10, and IFN-γ content by ELISA. The means ± SD from five independent experiments are shown.

Patients with X-linked autoimmunity-allergic dysregulation (XLAAD) who lack CD4⁺ CD25⁺ T cells are characterized by diabetes mellitus, high serum IgE levels, eosinophilia, and food allergy.²⁵ However, in most of the allergic individuals examined by us, CD4⁺ CD25⁺ Treg do not differ from those of non-atopic healthy controls as they both show intact regulatory functions and expression of CTLA-4 (intracellular), GITR and FoxP3 was present in the same quantity in peripheral blood as was observed for non-atopic healthy controls.¹⁸ Here we demonstrate that CD4⁺ CD25⁺ Treg from grass-pollen-allergic donors fail to inhibit proliferation but not cytokine production of CD4⁺ CD25⁻ responder T cells at high allergen concentrations while Treg from non-atopic donors retain their regulatory capacity even at these high concentrations. Additionally, we show that in the context of a strong allergen like wasp venom or phospholipase A₂ CD4⁺ CD25⁺ Treg from allergic and from healthy controls lose their regulatory function (proliferation and Th2 cytokine production) at high allergen doses and that the regulatory function was only retained in donors who were *not* allergic to wasp venom using low allergen doses.

Our observation concerning the loss of suppression at high antigen doses is in accordance with other studies showing that increased costimulation, activation with superantigens or addition of IL-2 breaks CD4⁺ CD25⁺ Treg-mediated suppression.^{22,26,27} Recently, George *et al.* analysed the mechanism whether under certain conditions CD4⁺ CD25⁺ Treg suppressor function is inactivated or whether increased stimulation causes responder T cells to escape suppression. They have demonstrated in a murine model with different T-cell receptor transgenic T cells that in the context of strong antigen-specific stimulation CD4⁺ CD25⁺ Treg remained partially functional because they still reduced cytokine levels and CD25 expression of Th cells in cocultures but they did no longer inhibited proliferation.²³ Thus, these results suggest that potently stimulated Th cells may partially escape Treg-mediated regulation, probably by producing high amounts of IL-2. In the allergologic context, the threshold of activation versus suppression may differ with the allergen exposure and the strength of the allergenic stimulus. Strong signals derived from foreign microbial antigens may activate T cells while target cells that receive weak signals, such as

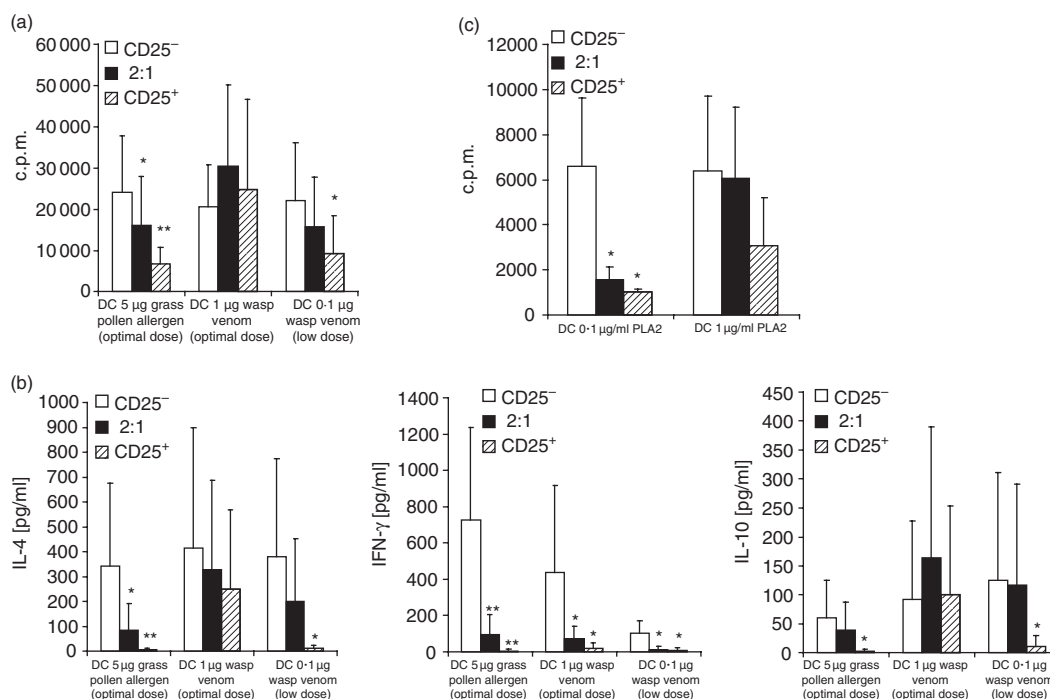


Figure 3. CD4⁺ CD25⁺ Treg suppress proliferation and Th2 cytokine production of CD4⁺ CD25⁻ T cells from donors who were *not* allergic to wasp venom only after stimulation with suboptimal doses of wasp-venom-pulsed DC. CD4⁺ CD25⁻ T cells (CD25⁻) cultured alone or in the presence of CD4⁺ CD25⁺ Treg (2 : 1) or Treg alone (CD25⁺) were stimulated with grass-pollen-allergen-pulsed, wasp-venom-pulsed, or phospholipase A₂-pulsed DC. (a, c) For analysis of proliferation [³H]TdR incorporation was measured at day 5. (b) For cytokine analysis the cocultures were restimulated with newly generated allergen-pulsed DC on day 7. Supernatants were collected 24 hr later and analysed for IL-4, IL-10, and IFN- γ content by ELISA. The means $\pm$ SD from 10 (for a and b) and six (for c) independent experiments are shown. * $P \leq 0.05$ and ** $P \leq 0.01$ represent statistically significant differences between CD4⁺ CD25⁻ T cells alone versus coculture with Treg or Treg alone. Data for wasp-allergic donors are shown in Figure 2.

environmental allergens, are suppressed.²⁷ This threshold theory, together with a potentially reduced function of regulatory cells in atopic/allergic patients, may explain why Th-cell proliferation from healthy donors was inhibited by CD4⁺ CD25⁺ Treg at all concentrations of grass pollen allergen while it was not inhibited in allergic donors at high concentrations. It may also explain the lack in regulation of Th cells from wasp-venom-allergic patients after stimulation with the strong allergen wasp venom even at low concentrations compared to healthy donors.

The finding that responder T cells from healthy donors did not escape CD4⁺ CD25⁺ Treg-mediated suppression at high allergen concentrations is in line with the recent report by Ling *et al.* who demonstrated that inhibition of allergen-driven proliferation and IL-5 production by CD4⁺ CD25⁺ Treg from allergic donors was less pronounced than that in healthy individuals, especially during the pollen season.²⁸ They also found that CD4⁺ CD25⁻ T cells from healthy donors produced greater amounts of IL-5 and IL-13 than unseparated PBMC, implying that Th2 cytokine production is usually inhibited by CD4⁺ CD25⁺ Treg. However, the fact that allergic diseases exist despite normal frequency and intact

function of CD4⁺ CD25⁺ Treg except at high allergen concentrations implies that additional mechanisms may be involved in their regulation. For example, IL-10-producing Tr1 cells are induced in the periphery together with a Th2 to Th1 shift during allergen immunotherapy.^{29,30} These Tr1 cells also express CD25 and were first described as preventing colitis in mice^{21,31,32} but CD25 expression by Tr1 cells may represent the activation status during coculture rather than a lineage marker of regulatory cells derived from the thymus.^{8,33} Investigations concerning the effect of specific immunotherapy on the regulatory function of CD4⁺ CD25⁺ Treg are ongoing. Furthermore, regulatory T cells can be induced by IL-10-treated DC.³⁴ We and others could show that these tolerogenic T cells are characterized by an inhibited proliferation and a reduced Th1 as well as Th2 cytokine production^{34,35} and that they produce high amounts of IL-10 (unpublished data).

Taken together, the regulation of allergic immune responses by CD4⁺ CD25⁺ Treg is dependent on the concentration and strength of the respective allergen as well as the allergic/atopic status of the donor. At high allergen concentrations the function of CD4⁺ CD25⁺ Treg from

grass-pollen-allergic donors is impaired compared to Treg from healthy individuals. In wasp-venom-allergic patients exposure to wasp venom always leads to activation of responder T cells which escape regulation by CD4⁺ CD25⁺ Treg, indicating the high allergenicity of these allergens (especially phospholipase A₂). Different thresholds concerning failure of regulation may therefore be correlated with the different allergenicity of different allergens on the one hand, and with the different regulatory capacity of different individuals strongly depending on their allergic/atopic status on the other hand. Threshold levels for allergen exposure leading to sensitization and exaggerated (uncontrolled, allergic) immune responses and allergic diseases may be related to the function of CD4⁺ CD25⁺ Treg and may vary for different allergens and for the individuals being exposed as is well-known from both clinical experience and epidemiological data.

Acknowledgements

This work was supported by the Deutsche Forschungsgemeinschaft (Sonderforschungsbereich 548 TP A4).

References

- Shevach EM. CD4⁺ CD25⁺ suppressor T cells: more questions than answers. *Nat Rev Immunol* 2002; **2**:389–400.
- Sakaguchi S. Naturally arising CD4⁺ regulatory T cells for immunologic self-tolerance and negative control of immune responses. *Annu Rev Immunol* 2004; **22**:531–62.
- McHugh RS, Shevach EM. The role of suppressor T cells in regulation of immune responses. *J Allergy Clin Immunol* 2002; **110**:693–702.
- Suri-Payer E, Cantor H. Differential cytokine requirements for regulation of autoimmune gastritis and colitis by CD4(+) CD25(+) T cells. *J Autoimmun* 2001; **16**:115–23.
- Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J Immunol* 1995; **155**:1151–64.
- Shimizu J, Yamazaki S, Takahashi T, Ishida Y, Sakaguchi S. Stimulation of CD25(+) CD4(+) regulatory T cells through GITR breaks immunological self-tolerance. *Nat Immunol* 2002; **3**:135–42.
- Wing K, Ekmark A, Karlsson H, Rudin A, Suri-Payer E. Characterization of human CD25⁺ CD4⁺ T cells in thymus, cord and adult blood. *Immunology* 2002; **106**:190–9.
- Levings MK, Sangregorio R, Sartirana C *et al.* Human CD25⁺CD4⁺ T suppressor cell clones produce transforming growth factor beta, but not interleukin 10, and are distinct from type 1 T regulatory cells. *J Exp Med* 2002; **196**:1335–46.
- Read S, Malmstrom V, Powrie F. Cytotoxic T lymphocyte-associated antigen 4 plays an essential role in the function of CD25(+) CD4(+) regulatory cells that control intestinal inflammation. *J Exp Med* 2000; **192**:295–302.
- Itoh M, Takahashi T, Sakaguchi N *et al.* Thymus and autoimmunity: production of CD25⁺CD4⁺ naturally anergic and suppressive T cells as a key function of the thymus in maintaining immunologic self-tolerance. *J Immunol* 1999; **162**:5317–26.
- Fontenot JD, Gavin MA, Rudensky AY. Foxp3 programs the development and function of CD4⁺CD25⁺ regulatory T cells. *Nat Immunol* 2003; **4**:330–6.
- Sakaguchi S. The origin of FOXP3-expressing CD4⁺ regulatory T cells: thymus or periphery. *J Clin Invest* 2003; **112**:1310–12.
- Jonuleit H, Schmitt E, Stassen M, Tuettenberg A, Knop J, Enk AH. Identification and functional characterization of human CD4(+) CD25(+) T cells with regulatory properties isolated from peripheral blood. *J Exp Med* 2001; **193**:1285–94.
- Dieckmann D, Plottner H, Berchtold S, Berger T, Schuler G. Ex vivo isolation and characterization of CD4(+) CD25(+) T cells with regulatory properties from human blood. *J Exp Med* 2001; **193**:1303–10.
- Levings MK, Sangregorio R, Roncarolo MG. Human CD25(+) CD4(+) T regulatory cells suppress naive and memory T cell proliferation and can be expanded *in vitro* without loss of function. *J Exp Med* 2001; **193**:1295–302.
- Walker MR, Kasprowicz DJ, Gersuk VH *et al.* Induction of FoxP3 and acquisition of T regulatory activity by stimulated human CD4⁺CD25⁺ T cells. *J Clin Invest* 2003; **112**:1437–43.
- Chen W, Jin W, Hardegen N *et al.* Conversion of peripheral CD4⁺CD25⁺ naive T cells to CD4⁺CD25⁺ regulatory T cells by TGF-beta induction of transcription factor Foxp3. *J Exp Med* 2003; **198**:1875–86.
- Bellinghausen I, Klostermann B, Knop J, Saloga J. Human CD4⁺CD25⁺ T cells derived from the majority of atopic donors are able to suppress TH1 and TH2 cytokine production. *J Allergy Clin Immunol* 2003; **111**:862–8.
- Cottrez F, Hurst SD, Coffman RL, Groux H. T regulatory cells 1 inhibit a Th2-specific response *in vivo*. *J Immunol* 2000; **165**:4848–53.
- Suto A, Nakajima H, Kagami SI, Suzuki K, Saito Y, Iwamoto I. Role of CD4(+) CD25(+) regulatory T cells in T helper 2 cell-mediated allergic inflammation in the airways. *Am J Respir Crit Care Med* 2001; **164**:680–7.
- Jutel M, Akdis M, Budak F *et al.* IL-10 and TGF-beta cooperate in the regulatory T cell response to mucosal allergens in normal immunity and specific immunotherapy. *Eur J Immunol* 2003; **33**:1205–14.
- Thornton AM, Shevach EM. CD4⁺CD25⁺ immunoregulatory T cells suppress polyclonal T cell activation *in vitro* by inhibiting interleukin 2 production. *J Exp Med* 1998; **188**:287–96.
- George TC, Bilsborough J, Viney JL, Norment AM. High antigen dose and activated dendritic cells enable Th cells to escape regulatory T cell-mediated suppression *in vitro*. *Eur J Immunol* 2003; **33**:502–11.
- Umetsu DT, Akbari O, DeKruyff RH. Regulatory T cells control the development of allergic disease and asthma. *J Allergy Clin Immunol* 2003; **112**:480–7.
- Wildin RS, Smyk-Pearson S, Filipovich AH. Clinical and molecular features of the immunodysregulation, polyendocrinopathy, enteropathy, X linked (IPEX) syndrome. *J Med Genet* 2002; **39**:537–45.
- Ou LS, Goleva E, Hall C, Leung DY. T regulatory cells in atopic dermatitis and subversion of their activity by superantigens. *J Allergy Clin Immunol* 2004; **113**:756–63.

- 27 Baecher-Allan C, Viglietta V, Hafler DA. Inhibition of human CD4(+) CD25(+high) regulatory T cell function. *J Immunol* 2002; **169** (11):6210–17.
- 28 Ling EM, Smith T, Nguyen XD, Pridgeon C, Dallman M, Arbery J *et al.* Relation of CD4⁺CD25⁺ regulatory T-cell suppression of allergen-driven T-cell activation to atopic status and expression of allergic disease. *Lancet* 2004; **363**:608–15.
- 29 Bellinghausen I, Metz G, Enk AH, Christmann S, Knop J, Saloga J. Insect venom immunotherapy induces interleukin-10 production and a Th2-to-Th1 shift, and changes surface marker expression in venom-allergic subjects. *Eur J Immunol* 1997; **27**:1131–9.
- 30 Akdis CA, Blesken T, Akdis M, Wüthrich B, Blaser K. Role of interleukin 10 in specific immunotherapy. *J Clin Invest* 1998; **102**:98–106.
- 31 Francis JN, Till SJ, Durham SR. Induction of IL-10⁺CD4⁺CD25⁺ T cells by grass pollen immunotherapy. *J Allergy Clin Immunol* 2003; **111**:1255–61.
- 32 Groux H, O'Garra A, Bigler M, Rouleau M, Antonenko S, de Vries JE, Roncarolo MG. A CD4⁺ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. *Nature* 1997; **389**:737–42.
- 33 Jordan MS, Boesteanu A, Reed AJ, Petrone AL, Hohenbeck AE, Lerman MA, Naji A, Caton AJ. Thymic selection of CD4⁺CD25⁺ regulatory T cells induced by an agonist self-peptide. *Nat Immunol* 2001; **2**:301–6.
- 34 Steinbrink K, Graulich E, Kubsch S, Knop J, Enk AH. CD4(+) and CD8(+) anergic T cells induced by interleukin-10-treated human dendritic cells display antigen-specific suppressor activity. *Blood* 2002; **99**:2468–76.
- 35 Bellinghausen I, Brand U, Steinbrink K, Enk AH, Knop J, Saloga J. Inhibition of human allergic T cell responses by interleukin-10-treated dendritic cells: differences from hydrocortisone-treated dendritic cells. *J Allergy Clin Immunol* 2001; **108**: 242–9.