

Wasp venom immunotherapy induces activation and homing of CD4⁺CD25⁺ forkhead box protein 3–positive regulatory T cells controlling T_H1 responses

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Background: Despite a growing interest in CD4⁺CD25⁺ forkhead box protein 3 (Foxp3)–positive regulatory T (Treg) cells, the fundamental parameters of the activation and homing of these cells during wasp venom immunotherapy (VIT) are largely unknown.

Objective: We investigated longitudinally the phenotype and function of Treg cells in a well-characterized homogeneous group of patients with wasp venom allergy during VIT.

Methods: In 30 patients peripheral Treg cells were *ex vivo* monitored for their activation status and homing capacities by means of flow cytometric analysis before and after 1 and 6 months of VIT. In addition, the *in vitro* suppressive activity of Treg cells, as well as cytokine secretion, in response to wasp venom was analyzed.

Results: One month after initiating VIT, the proportion of both CD4⁺CD25⁺Foxp3⁺ and CD4⁺Foxp3⁺ Treg cells significantly decreased in peripheral blood. Coexpression of the lymph node homing receptors CCR7/CD62L were induced in CD4⁺Foxp3⁺CD45RO⁺ Treg cells, indicating recirculation of VIT-activated Treg cells in secondary lymphoid organs. *In vivo* imaging by means of color duplex ultrasonography of the axillary draining lymph nodes demonstrated a VIT-induced 4-fold augmentation in afferent arterial blood flow.

Furthermore, increased activation markers (CD45RO and HLA-DR) of Treg cells correlated with effective *in vitro* suppression of wasp venom–driven T-cell proliferation. After 1 month of VIT, Treg cell depletion *in vitro* greatly enhanced wasp venom–induced IFN- γ secretion.

Conclusions: Allergen exposure during VIT simultaneously induces the activation and selective homing of circulating Treg cells. Functionally, on the one hand, Treg cells balance the immune reaction toward tolerance, and on the other

hand, they are involved in controlling overwhelming T_H1 responses. (J Allergy Clin Immunol 2010;■■■■:■■■■–■■■■.)

Key words: Immunotherapy, lymph node, regulatory T cell, tolerance, wasp venom

Venom immunotherapy (VIT) of wasp sting allergy by means of subcutaneous injection of high venom doses is one of the clinically most effective forms of immunotherapy. Proposed mechanisms include a modulation of T- and B-cell responses and related antibody isotypes, namely a decrease in venom-specific IgE levels and induction of venom-specific IgG, especially allergen-blocking IgG4.^{1–3} The T_H2-dominated immune response is balanced toward a T_H1 response, leading to decreased IL-4 but increased IFN- γ responses by allergen-specific T cells.^{4–7} Regulatory T (Treg) cells are believed to be important for the successful outcome of VIT because VIT-treated patients have been shown to exhibit suppressed proliferative responses accompanied by increased levels of IL-10 in the supernatants of venom-stimulated T-cell cultures.^{7–9} Furthermore, the activity of both IL-10–secreting Treg cells and CD4⁺CD25⁺ Treg cells is compromised in patients with allergic disease.^{10,11} However, studies longitudinally monitoring the activation and homing capacities of the human CD4⁺CD25⁺ forkhead box protein 3 (Foxp3)–positive Treg cell compartment during VIT are missing.

Therefore we performed a longitudinal study on Treg cell phenotype and function during VIT in a large, well-characterized, and homogeneous group of patients with wasp sting allergy.

METHODS

Subjects

Thirty patients with a history of severe anaphylaxis to wasp stings were studied. After diagnosis, induction of wasp VIT was carried out during 3 consecutive days, in which escalating venom doses were injected subcutaneously at the upper arms. Doses were increased from 0.1 to 100 μ g, reaching a total amount of 201.1 μ g of venom on day 3. Subsequently, maintenance injections of 100 μ g of aluminum hydroxide–adsorbed venom were given after 2 days and 1, 4, and 8 weeks and thereafter for every 6 weeks. Blood samples were drawn at the start and both 1 month and 6 months after initiation of wasp VIT before the injection of venom. Healthy, nonatopic, and nonallergic subjects were used for control experiments. Informed consent was obtained from all subjects, and the study was approved by the Ethical Committee Board of the University of Würzburg, Germany. All clinical investigations were conducted according to Declaration of Helsinki principles. See the Methods section of this article's Online Repository at www.jacionline.org for more information.

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Abbreviations used

Foxp3: Forkhead box protein 3
Treg: Regulatory T
TT: Tetanus toxoid
VIT: Venom immunotherapy

RESULTS**Patients**

Sixteen male patients (mean age, 39 years; age range, 21–56 years) and 14 female patients (mean age, 43 years; age range, 24–68 years) with a history of anaphylaxis caused by wasp stings within the last 2 years who were eligible for VIT according to the criteria of the European Academy of Allergy and Clinical Immunology¹² were studied. Specific sensitization was confirmed based on positive skin test results (prick and intradermal tests) and detection of allergen-specific serum IgE levels. Patients' clinical characteristics are summarized in Table E1 (available in this article's Online Repository at www.jacionline.org). VIT was carried out as described in the Methods section, and blood samples were drawn as shown in Fig 1.

Transient decrease in Treg cell numbers

We first investigated whether the number of CD4⁺CD25⁺ T cells was altered in the longitudinal course of VIT. Because previous studies have shown that only the CD4⁺ T-cell population with the highest CD25 expression exerts suppressive Treg cell activity,¹³ we used the gating approach, as shown in Fig E1 (available in this article's Online Repository at www.jacionline.org). In the peripheral blood frequencies of CD4⁺CD25⁺ and CD4⁺CD25^{hi} T cells remained comparably stable throughout the 6 months of VIT with a slight but not significant decrease in CD4⁺CD25⁺ and CD4⁺CD25^{hi} populations 1 month after starting therapy (CD4⁺CD25⁺ mean $\pm$ SEM: 4.1% $\pm$ 0.2 compared with 3.6% $\pm$ 0.28; CD4⁺CD25^{hi} mean $\pm$ SEM: 1.73% $\pm$ 0.11% compared with 1.47% $\pm$ 0.14; Fig 2, A, and see Fig E2 in this article's Online Repository at www.jacionline.org). Surprisingly, the proportion of Foxp3⁺ Treg cells in the CD25⁺ and CD25^{hi} T-cell compartment displayed a significant decrease 1 month after initiating VIT (CD4⁺CD25⁺Foxp3⁺ mean $\pm$ SEM: 62.2% $\pm$ 2.22% compared with 44.3% $\pm$ 3.5%; CD4⁺CD25^{hi}Foxp3⁺ mean $\pm$ SEM: 77.9% $\pm$ 2.11% compared with 57.9% $\pm$ 4.74%; Fig 2, B). Because the described gating approach might underestimate the frequency of Treg cells,^{14,15} the Foxp3 expression of CD4⁺ gated T cells was additionally analyzed on the basis of the CD25 marker (Fig 3, A). Using this method, we also observed a lower proportion of Treg cells in the peripheral blood after 1 month of VIT, which returned to baseline levels after 6 months (mean $\pm$ SEM: 5.7% $\pm$ 0.23% compared with 4.4% $\pm$ 0.33% and 5.6% $\pm$ 0.6%, respectively; Fig 3, B, and see Fig E3 in this article's Online Repository at www.jacionline.org).

Concomitant activation of Treg cells

Next we determined CD4⁺Foxp3⁺ and CD4⁺Foxp3[−] populations for CD45RO, HLA-DR, and Ki-67 expression. Whereas the frequencies of CD45RO⁺ cells within the CD4⁺Foxp3[−] T-cell compartment slightly increased 1 month after starting VIT (mean $\pm$ SEM: 41.2% $\pm$ 3.1% compared with 43.6% $\pm$ 2.8%,

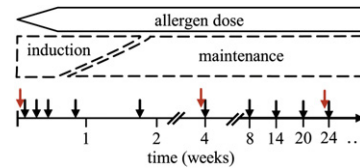


FIG 1. Induction and maintenance of wasp VIT. Red arrows designate the drawing of blood samples before the subcutaneous injection of venom (black arrows).

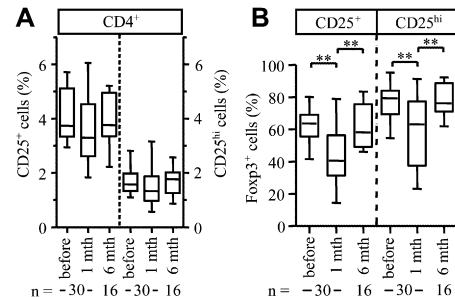


FIG 2. Peripheral CD25⁺ T cells along wasp VIT. **A**, Frequencies of CD25⁺ and CD25^{hi} cells. **B**, Percentages of CD25⁺Foxp3⁺ and CD25^{hi}Foxp3⁺CD4⁺ T cells. Statistical analysis was performed in 16 longitudinally monitored patients (see Fig E2 in this article's Online Repository). ***P* < .01.

P > .05), the relative proportion of CD45RO-expressing Treg cells within the CD4⁺Foxp3⁺ T-cell subset significantly increased during this time period (mean $\pm$ SEM: 71.5% $\pm$ 2.3% compared with 77.1% $\pm$ 2.1%, *P* < .05; Fig 3, C). CD4⁺Foxp3⁺CD45RO⁺ Treg cells were largely CD127^{low} and CD127[−] (Fig 3, D), excluding that recently activated CD4⁺CD25[−] effector T cells transiently upregulated Foxp3.^{16–18}

Treg cell turnover

Parallel with the induction of CD45RO on CD4⁺Foxp3⁺ Treg cells, enhanced HLA-DR expression on CD45RO⁺ Treg cells (n = 5; mean $\pm$ SEM: 39.4% $\pm$ 1.93% compared with 1 month: 45.8% $\pm$ 0.88%, *P* = .041; Fig 3, E) and CD45RO[−] Foxp3[−] T cells (n = 5; mean $\pm$ SEM: 5.5% $\pm$ 0.77% compared with 1 month: 6.7% $\pm$ 0.81%, *P* = .04) in VIT-treated patients but not healthy control subjects (n = 4; CD45RO⁺Foxp3[−] T cells mean $\pm$ SEM: 7.2% $\pm$ 1.25% compared with 1 month: 8.1% $\pm$ 1.52%, *P* = .08; CD45RO[−] Treg cells: 40.6% $\pm$ 2.89 compared with 1 month: 41.7% $\pm$ 2.67%, *P* = .08; data not shown) could be observed. HLA-DR expression was induced in CD45RO⁺ Treg cells and CD45RO[−]Foxp3[−] T cells at the same relative ratio (n = 5; mean $\pm$ SEM: 1.18 $\pm$ 0.07 compared with 1.19 $\pm$ 0.08). As shown recently,^{19,20} these findings suggest that Foxp3⁺ Treg cells might be derived from the conventional CD45RO⁺Foxp3[−] T-cell pool by means of repetitive antigen challenge. Therefore we measured the proliferating Treg cell fraction using Ki-67 expression, a nuclear protein expressed in all phases of the active cell cycle.²¹ In line with findings by others,^{19,20} Ki-67 expression of Treg cells was higher compared with expression of CD45RO⁺Foxp3[−] T cells but did not change significantly in either group 1 month after starting VIT (n = 5; CD45RO⁺ Treg cells mean $\pm$ SEM: 25.4% $\pm$ 2.26% compared with 1 month: 27.5% $\pm$ 2.24%, *P* = .5; CD45RO[−]Foxp3[−] T cells mean $\pm$ SEM: 6.4% $\pm$ 1.3% compared with 1 month: 5.9% $\pm$ 1.0%,

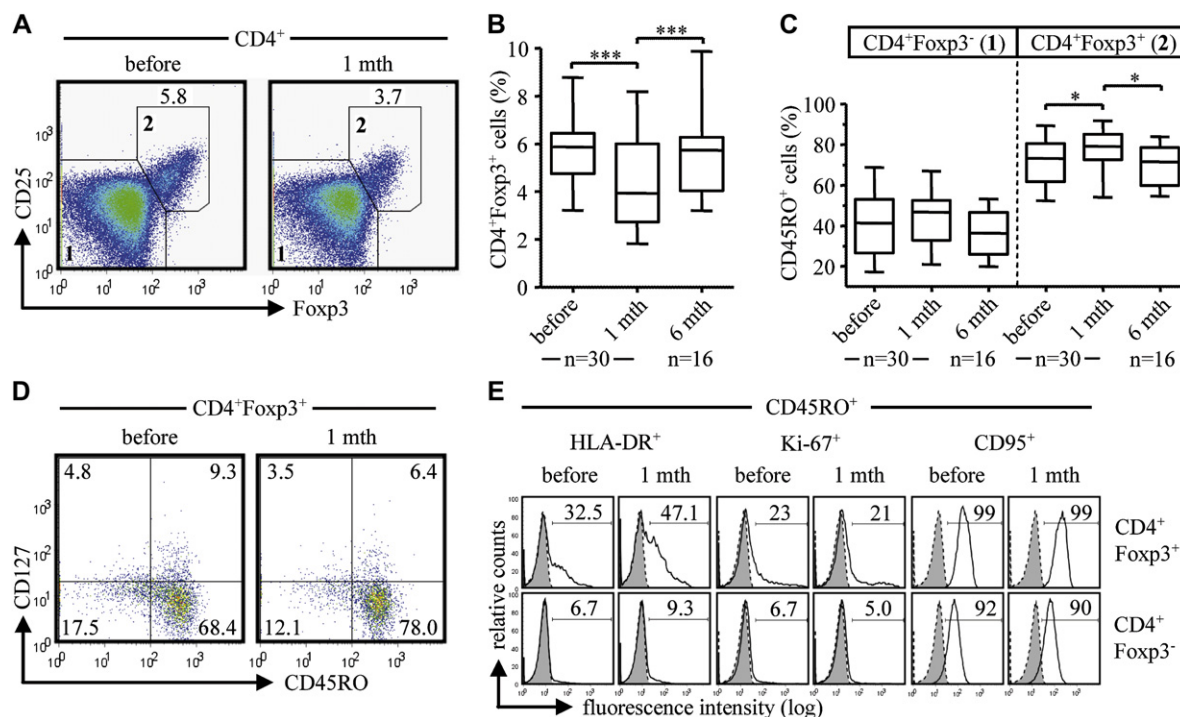


FIG 3. Activation of Treg cells. **A**, Dot plots of CD4⁺ Foxp3⁺ Treg cells (gate 2). **B**, Decrease in CD4⁺ Foxp3⁺ Treg cell numbers. ****P* < .001. Statistical analysis was performed as described in Fig 2 (see Fig E3 in this article's Online Repository). **C**, CD45RO expression of CD4⁺ Foxp3⁻ (gate 1) and CD4⁺ Foxp3⁺ (gate 2) cells. **P* < .05. **D**, CD127 expression of Treg cells. **E**, Markers of activation and CD95 expression of Treg cells.

P = .4; Fig 3, E). Because Treg cell proliferation might be terminated by CD95-induced apoptosis,²² we next investigated the expression of this death receptor. Treg cells indeed constitutively express CD95 to a higher degree compared with CD45RO⁺ Foxp3⁻ T cells (Fig 3, E). However, CD95 expression levels of Treg cells did not change during 1 month of VIT (*n* = 5; mean fluorescence intensity ± SEM: 210.4 ± 16.19 compared with 1 month: 220.2 ± 18.57, *P* = .46).

Expression of lymph node homing receptors on Treg cells

Induction of peripheral antigen-specific Treg cells critically depends on the capacity to enter secondary lymphoid tissues, where Treg cells exert their suppressive function *in vivo*.^{23,24} We therefore analyzed the adhesion and homing molecules CD62L, CD11a (lymphocyte function-associated antigen 1), and CCR7 on memory (CD4⁺ CD25⁺ Foxp3⁺ CD45RO⁺) and naive (CD4⁺ CD25⁺ Foxp3⁺ CD45RA⁺) Treg cells, as well as conventional memory (CD4⁺ CD25⁻ Foxp3⁻ CD45RO⁺) and naive (CD4⁺ CD25⁻ Foxp3⁻ CD45RA⁺) T cells. Whereas CD62L and CD11a expression levels were not altered within the various T-cell subsets in the time course of VIT, VIT induced the relative proportion of CCR7⁺ memory Treg cells after 1 and 6 months (mean ± SEM: 38.8% ± 3.3% compared with 51.1% ± 3.8% and 57.3% ± 4.7%, respectively; see Fig E4 in this article's Online Repository at www.jacionline.org). Concurrently, CCR7 expression moderately increased on memory T cells (mean ± SEM: 71.9% ± 3.1% compared with 78.1% ± 2.8% and 81.8% ± 4.5%). Both CCR7 and CD62L expression are markers for lymphocyte migration to lymph nodes.²⁵ Interestingly, VIT

significantly induced coexpression of CCR7 and CD62L on memory Treg cells (mean ± SEM: 33.7% ± 2.9% compared with 44.9% ± 3.3% and 56.0% ± 3.9%; Fig 4, A), whereas memory T cells displayed an unaltered but higher coexpression of these molecules than memory Treg cells 1 and 6 months after initiating VIT (mean ± SEM: 70.2% ± 3.4% compared with 70.0% ± 3.3% and 74.8% ± 4.3%, respectively).

Increased blood flow in draining lymph nodes

Immune responses elicited by means of VIT are believed to take place in the regional draining lymph nodes. Therefore we performed *in vivo* imaging of axillary lymph nodes by means of color duplex ultrasonography. This technique demonstrated a 4-fold increase in afferent arterial lymph node blood flow early after initiation of VIT (*n* = 4; mean ± SEM: 1.4 ± 0.12 mL/min compared with 5.2 ± 1.13 mL/min; Fig 4, B). The transient decrease in Treg cell numbers in peripheral blood together with the increased blood flow through draining lymph nodes argues for a homing of activated Treg cells to secondary lymphoid organs.

Reduced wasp venom-driven T-cell proliferation

To determine whether activation (as measured by CD45RO and HLA-DR expression) and maintained proliferation (as measured by Ki-67 expression) of Treg cells during a continuous allergen challenge translates into suppressive activity, we next performed *in vitro* proliferation assays. Freshly isolated PBMCs were stimulated with different concentrations of wasp venom or tetanus toxoid (TT) as a recall antigen. As shown in Fig 5, A, proliferation induced by wasp venom was readily detected before initiation of

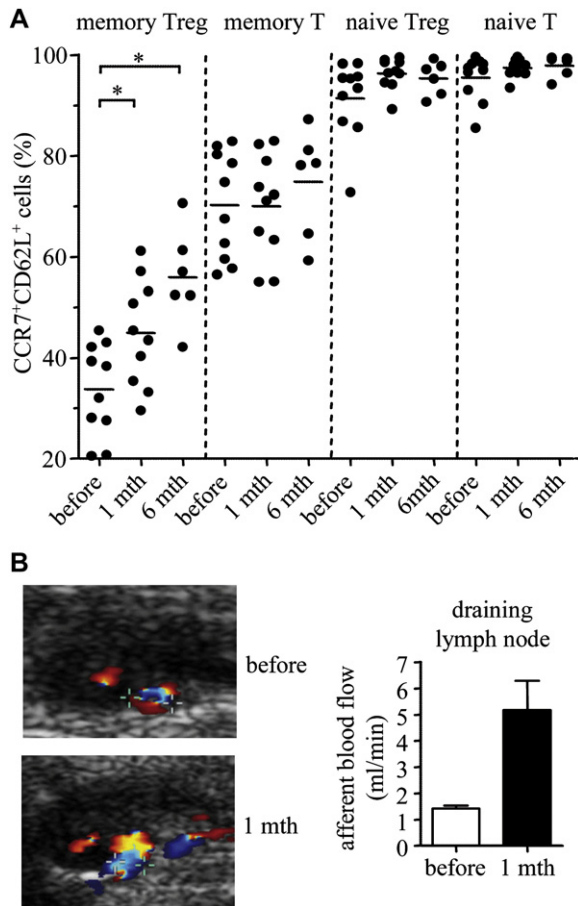


FIG 4. Expression of T-cell homing molecules. **A**, CCR7/CD62L coexpression. Individual dots and means (horizontal line) of T-cell populations shown are indicated. * $P < .05$ in 6 longitudinally followed patients. **B**, Color duplex ultrasonography of axillary lymph nodes.

VIT, reaching a stimulation index of approximately 3 at 1 $\mu\text{g/mL}$ wasp venom. Strikingly, in correlation with the activated phenotype of Treg cells, proliferative responses to wasp venom were abolished 1 month after the onset of VIT, recovering to even higher proliferation rates after 6 months of VIT. TT responses remained stable throughout the longitudinal time course of VIT, indicating that the differences in T-cell proliferation elicited by wasp venom can be accounted to VIT.

Reciprocal IL-10 and IFN- γ secretion

Although stimulation of PBMCs with TT induced comparable levels of IFN- γ in supernatants over a period of 6 months, VIT did not induce wasp venom-driven IFN- γ production before 6 months of therapy (Fig 5, B). Conversely, wasp venom-induced IL-10 production was most pronounced after 1 month of VIT, returning virtually to baseline levels after 6 months of therapy. Using our assay system and also ELISPOT assays, we were not able to detect IL-4 or TGF- β in the course of VIT (data not shown).

Treg cells suppress wasp venom-driven proliferation

To investigate the suppressive activity of Treg cells, we depleted PBMCs from CD25⁺ cells before and after 1 month of VIT,

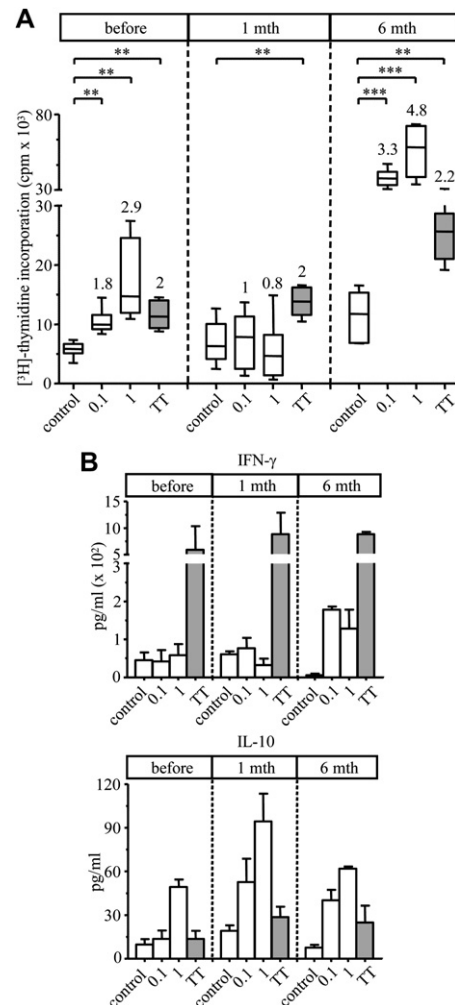


FIG 5. Proliferation and cytokine secretion. **A**, Stimulation indices induced by wasp venom (0.1 and 1 $\mu\text{g/mL}$) or TT (1 $\mu\text{g/mL}$). Triplicates of 6 (before, 1 month) and 3 (6 months) representative patients are shown. ** $P < .01$ and *** $P < .001$. **B**, IFN- γ and IL-10 measurements of 6 (before, 1 month) and 3 (6 months) representative patients (mean $\pm$ SEM).

followed by proliferation analysis and IFN- γ and IL-10 measurement (Fig 6, A). Electromagnetic separation resulted in a highly purified CD4⁺Foxp3⁺ population (<1% Foxp3⁺ cells in the remaining CD4⁺CD25^{low} population, data not shown). As shown in Fig 6, B, depleting CD25⁺ cells had no significant effect on wasp venom-driven proliferation before starting VIT. In contrast, after 1 month of VIT wasp venom-induced abrogation of T-cell proliferation was completely reconstituted in CD25⁺-depleted cell cultures. Moreover, proliferation rates elicited by 1 $\mu\text{g/mL}$ wasp venom were even higher than before the start of VIT, as indicated by the stimulation index (stimulation index, 4.8 vs 2.9).

Treg cells suppress wasp venom-induced IFN- γ secretion

IFN- γ production significantly increased in wasp venom-stimulated cell cultures depleted from CD25⁺ cells, indicating that in the absence of CD25⁺ cells, wasp venom-driven IFN- γ responses are uncontrolled (Fig 6, C). Before the start of VIT, IFN- γ secretion was also enhanced in nonstimulated cultures devoid of

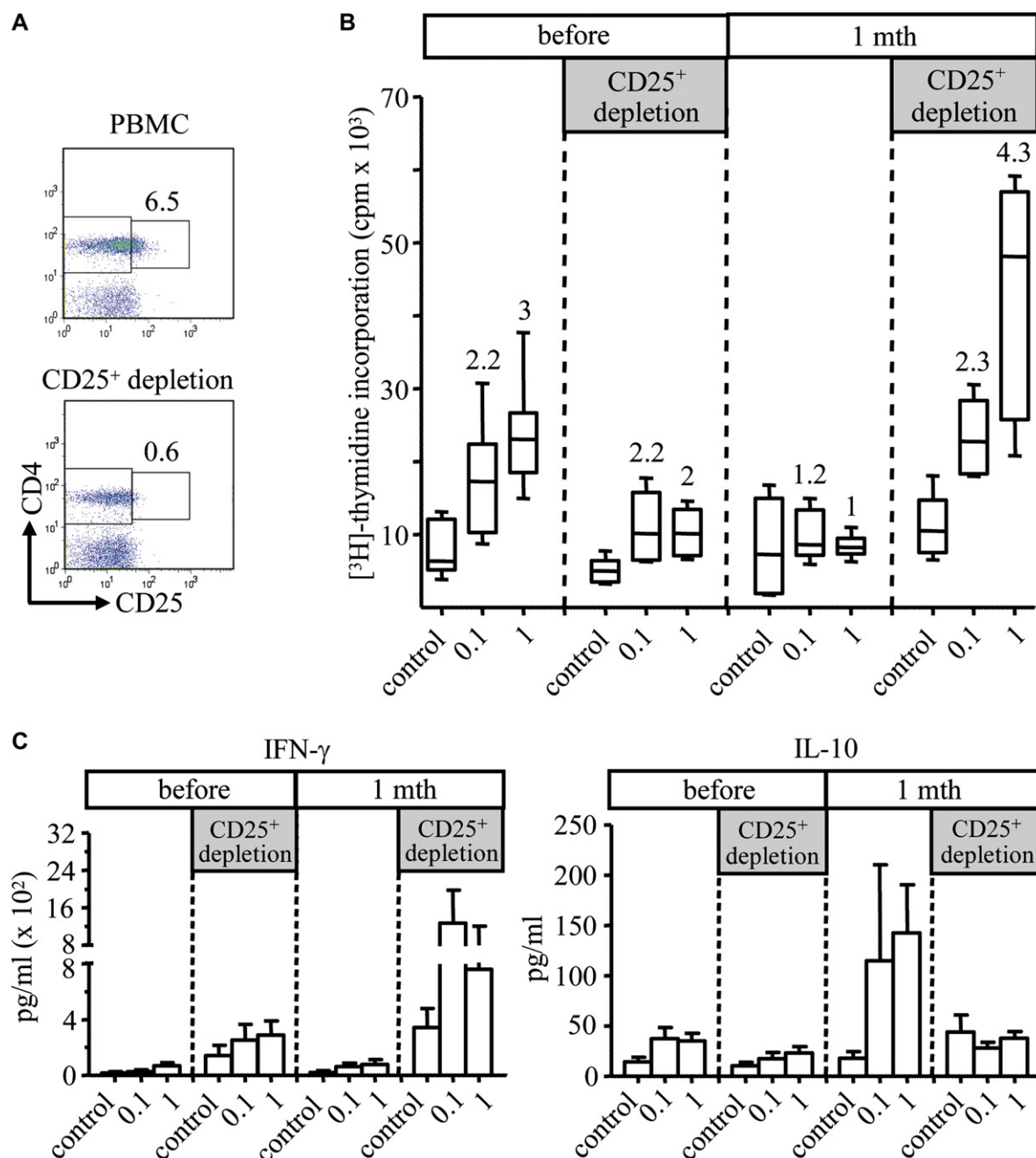


FIG 6. Treg cell depletion restores proliferation induced by wasp venom after 1 month of VIT. **A**, Numbers indicate the percentage of CD25⁺ cells after depletion. **B**, Stimulation indices of proliferation induced by wasp venom. **C**, IFN-γ and IL-10 measurements of 3 representative patients (mean ± SEM).

CD25⁺ cells. This “spontaneous” IFN-γ secretion was even enhanced in the course of VIT, suggesting that in the process of ongoing allergen challenge and in the absence of Treg cells, conventional T cells are prone to recognize yet unidentified autologous antigens during *in vitro* culture. In contrast, IL-10 production was abrogated in wasp venom-stimulated, CD25⁺-depleted cell cultures 1 month after initiation of VIT.

DISCUSSION

Induction of tolerance by immunotherapy has been associated with Treg cells, either by demonstrating an enhanced IL-10

production in cell culture or an increase in the CD4⁺CD25^{hi} population in peripheral blood. Here we determined the phenotype and function of CD4⁺CD25⁺Foxp3⁺ Treg cells during the longitudinal course of VIT in patients with wasp venom allergy diagnosed by using a standardized allergologic workup. One month after initiating VIT, a transient decrease in Treg cell numbers was accompanied by an enhanced activation and lymph node homing phenotype. During this initial phase of VIT, wasp venom-induced IL-10 secretion increased, whereas wasp venom-stimulated proliferation ceased. Importantly, IFN-γ production seemed to be controlled by wasp venom-induced Treg

cells, thereby dampening a potentially harmful exaggerated IFN- γ response.

Expression of CCR7 and CD62L is essential for normal lymphoid architecture and trafficking because CCR7- or CD62L-deficient mice have disturbed lymphoid architecture and impaired immune responses.²⁶ CCR7-deficient Treg cells do not migrate into the T-cell zone of lymph nodes. In memory T cells we detected an enhanced CCR7 expression during the early phase of VIT and a stably high coexpression of CCR7/CD62L throughout the course of VIT. Naive Treg and T cells displayed an approximately 100% expression of these receptors, as previously reported.^{27,28} It is hypothesized that CCR7⁺ (central) memory T cells, which have already encountered antigen, mainly reside in T-cell zones of lymph nodes to proliferate on re-encountering antigen.²⁹ The VIT-mediated induction of CCR7⁺CD62L⁺ Treg cells might indicate homing of Treg cells in secondary lymphoid organs to suppress T-cell effector responses. Concomitantly, afferent blood flow in axillary draining lymph nodes increased, indicating that regional lymph nodes are involved in subcutaneously administered VIT. In contrast to classical immunization procedures, such as diphtheria-pertussis-tetanus or poliomyelitis, VIT is not associated with clinically detectable lymphadenopathy (lymph node size determined by means of ultrasonography remained constant during VIT, data not shown) but sufficient to increase the cellular flow through in regional lymph nodes. These findings underline recent advances of immunotherapy by means of intranodal allergen injections.³⁰

Late cutaneous reactions around the injection sites regularly occur during initial VIT but usually vanish in the maintenance phase. The same clinical phenomenon can be observed after repeated seasonal bee stings in beekeepers.³¹ Therefore one intriguing possibility would be that at the onset of VIT, Treg cells get first educated in the skin and then migrate to the lymph nodes, as proposed in an allotransplantation model.³² Because human Treg cells have skin-homing capacities through the expression of CCR4 and CLA (data not shown),^{28,33} such a scenario might also hold true for VIT. Interestingly, during the first week of VIT, when late cutaneous reactions occur, peripheral numbers of CD4⁺CD25⁺Foxp3⁺ Treg cells did not decrease nor was an increase in CCR7⁺CD62L⁺ Treg cells detected (data not shown). An increase in IL-10⁺ and CD4⁺CD25⁺ cells has been demonstrated in the cutaneous inflammatory infiltrate at injection sites of immunotherapy.^{34,35} However, future studies have to clarify the potential migration of Treg cells from peripheral inflamed tissue (the late cutaneous reaction) to draining lymph nodes.

Immunotherapy-induced immune deviation toward T_H1 responses protects against allergic disease because IFN- γ inhibits the differentiation of T_H2 and IgE-secreting B cells.³⁶ However, in mice T_H1 cells cause potentiation of rather than protection against T_H2-mediated lung inflammation.³⁷ Furthermore, it has been reported that PBMCs depleted from CD25⁺ cells and stimulated with hepatitis C virus antigens led to a pronounced increase in proliferation and IFN- γ production in nonviremic, hepatitis C virus-infected patients, supporting the concept that Treg cells minimize the potential bystander damage associated with a potent antiviral T_H1 response.³⁸ Accordingly, we found that after 1 month of VIT, the wasp venom-induced IFN- γ response was greatly enhanced in Treg cell-depleted cell cultures. We hypothesize that Treg cells suppress potentially harmful VIT-induced exaggerated IFN- γ responses that are elicited by repetitive high-dose exposure to wasp venom allergens.

In addition to the effects of immunotherapy on immunoglobulins, its effects on T cells is of central importance. Some peripheral blood studies have indicated a shift in the balance of T-cell subsets toward a T_H1 phenotype because of preferential production of IFN- γ and decreased production of IL-4 and IL-5. However, these findings are not consistent. The consistent finding is an increased allergen-induced IL-10 secretion during early immunotherapy. The results of our study emphasize the importance of Treg cells for the therapeutic effects of immunotherapy. Moreover, the data suggest that Treg cells control a potentially harmful exaggerated IFN- γ response.

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Key Messages

- In the early phase of wasp VIT, a transient decrease in peripheral CD4⁺CD25⁺Foxp3⁺ Treg cell numbers is accompanied by an activated phenotype and induction of CCR7/CD62L coexpression in the circulating Treg cell population.
- The unexpected decrease in peripheral Treg cell numbers might be explained by enhanced CCR7/CD62L coexpression, suggesting a VIT-induced Treg cell recirculation into secondary lymphoid organs.
- The circulating CD4⁺CD25⁺Foxp3⁺ Treg cells efficiently suppressed proliferative responses to wasp venom, as well as IFN- γ production by conventional T cells.
- Treg cells seem to play a dual role during VIT: balancing the immune reaction toward tolerance and controlling an overwhelming and potentially harmful T_H1 response.

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METHODS

Skin testing

Sensitivity to wasp venom was determined by means of intradermal skin testing with increasing concentrations of wasp venom (ALK-Scherax, Hamburg, Germany) ranging from 0.001 to 1 $\mu\text{g/mL}$.

Serum IgE measurement

Serum total IgE and venom-specific IgE levels were determined by using the ImmunoCAP method (Phadia, Uppsala, Sweden), according to the manufacturer's instructions.

Antibodies and FACS analysis

The phenotype of the T cells was defined by means of flow cytometry with the following mAbs: CD4–peridinin-chlorophyll-protein (SK 3), CD25–allophycocyanin–Cy7 (M-A251), CD45RO–phycoerythrin–Cy7 (UCHL-1), CD62L–phycoerythrin (DREG-56), CD11a–fluorescein isothiocyanate (HI111), HLA-DR–fluorescein isothiocyanate (G46-6), Ki-67–phycoerythrin (B56), CD95–phycoerythrin (DX2; all from Becton Dickinson, Heidelberg, Germany), Foxp3–allophycocyanin (PCH101; NatuTec, Frankfurt, Germany), and CCR7–fluorescein isothiocyanate (150503; R&D Systems, Wiesbaden, Germany). Appropriate isotype controls were purchased from Becton Dickinson. Duplicate or triplicate patient samples were acquired on a FACSCanto (Becton Dickinson) and analyzed with FlowJo 7.22 software (Tree Star, Inc, Ashland, Ore). Stained PBMCs were first gated on CD4⁺ cells and then analyzed for the expression of CD25 and Foxp3 to determine T cells positive for Foxp3. Gates of CD4⁺CD25⁺ cells were set according to the isotype control, and gates of CD4⁺CD25^{hi} populations were set according to the low CD25⁺ staining of CD8⁺ cells (CD4⁺ cells), as described previously.^{E1} CD4⁺CD25⁺CD45RO⁺Foxp3⁺ cells were defined as memory Treg cells, and CD4⁺CD25⁺CD45RO⁺Foxp3⁺ cells were defined as naive Treg cells.^{E2} For clarity, CD45RO⁺ populations were designated as CD45RA⁺.

Cell isolation, culture, and stimulation

PBMCs were obtained from heparinized blood with Ficoll (Linaris, Wertheim, Germany) density centrifugation. For CD25⁺ depletion, PBMCs were incubated with CD25 microbeads (10 $\mu\text{L}/10^7$ cells; Miltenyi Biotec, Bergisch Gladbach, Germany) for 15 minutes, and cell fractions were separated on a LD column (Miltenyi Biotec). Cells were cultured in 96-well round-bottomed plates by using RPMI 1640 supplemented with 5% autologous serum, 2 nmol/L L-glutamine, 1 mmol/L sodium pyruvate, and nonessential amino acids (100:1 vol/vol; all from Gibco Invitrogen, Karlsruhe, Germany). Freshly isolated PBMCs (5×10^5) were incubated with the

indicated concentrations of wasp venom (ALK-Scherax) or TT (Merck, Darmstadt, Germany) in the presence of 30 U/mL IL-2 (Miltenyi Biotec) for 7 days. Proliferation was determined by pulsing triplicate cultures with tritiated thymidine (0.5 $\mu\text{Ci/mL}$; Amersham Biosciences, Braunschweig, Germany) 16 hours before harvesting. The stimulation index was calculated by dividing mean counts per minute of stimulated cultures by mean counts per minute of unstimulated cultures.

Cytokine measurement

For determination of IFN- γ , IL-4, and IL-10 secretion, 10 μL of cell-culture supernatant was obtained from triplicate cultures on day 6 of stimulation and subjected to the Cytometric Bead Array–based immunoassay (Becton Dickinson), allowing the simultaneous detection of these cytokines.

Duplex ultrasonography of axillary lymph nodes

High-resolution color-coded Duplex ultrasonography (Acuson Sequoia, Siemens, Germany) was performed with a 14-5 MHz linear-type transducer, allowing assessment of the local blood flow of the vasa afferentia of the lymph nodes. Analysis was performed before and 1 month after initiating wasp VIT on the unilateral axillary draining lymph node by using the same settings (Doppler frequency, wall filter, persistence, and pulse repetition frequency). The angle-corrected flow velocities of 1 axillary lymph node were measured 3 times by using the pulsed-wave Doppler mode, and the maximal mean flow velocities through the vessel were determined.

Statistical analysis

Box-and-whisker plots used indicate the minimum value, lower quartile, median, upper quartile, and maximum value. Longitudinal data were first subjected to repeated-measures ANOVA, followed by the Tukey-Kramer test for comparisons of all pairs. If data were only available for 2 time points (before and after 1 month), a paired *t* test was used. Otherwise, unpaired *t* tests were performed (GraphPad Prism 5.0; GraphPad Software, Inc, La Jolla, Calif). *P* values of less than .05 were considered significant.

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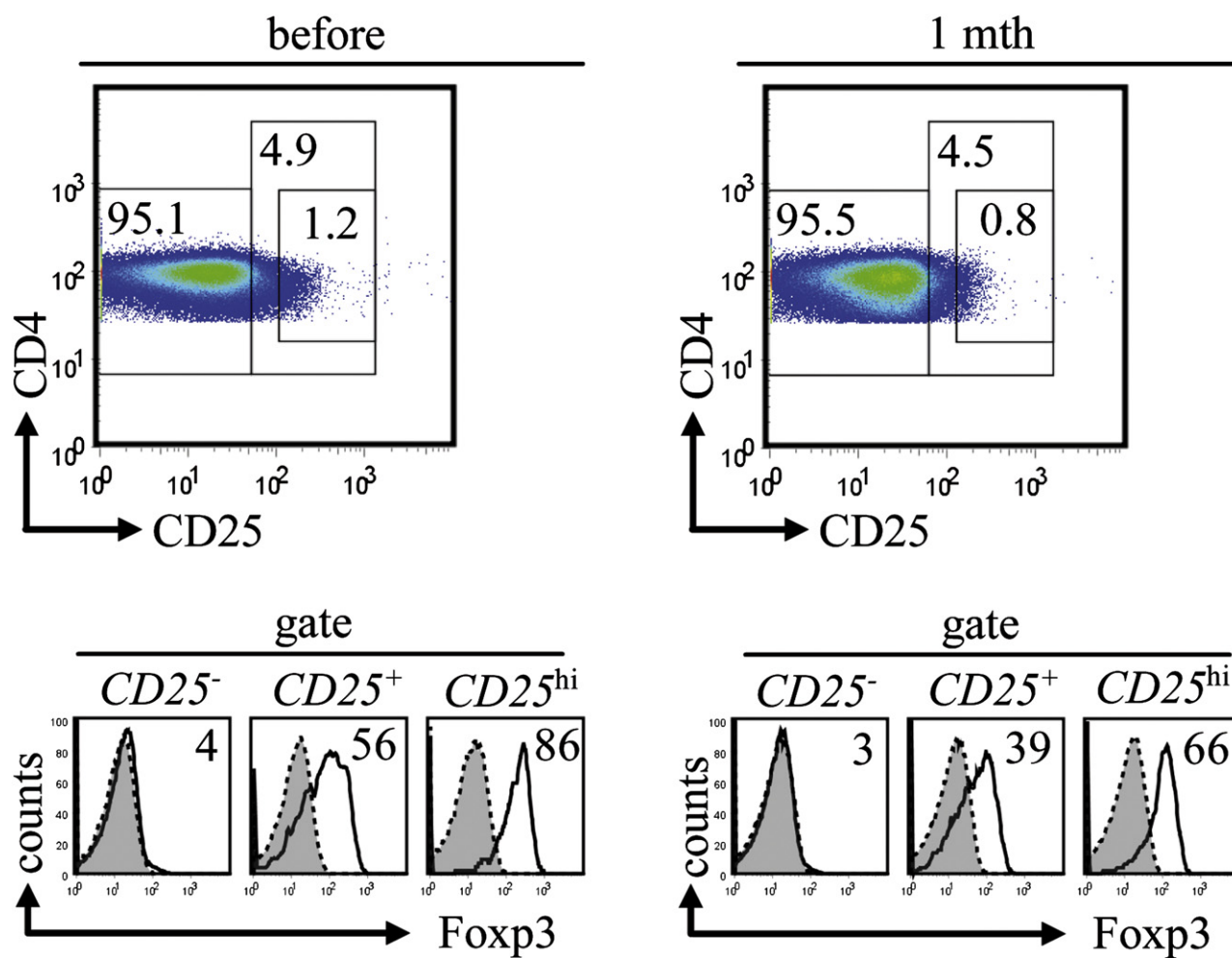


FIG E1. Gating strategy. Treg cells are enriched in the $CD4^+CD25^{hi}$ population. Histograms illustrate percentages of Foxp3⁺ cells in gated populations.

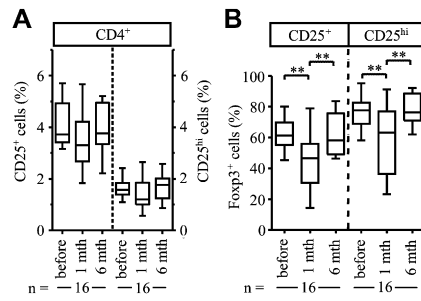


FIG E2. CD25⁺ T cells during VIT in 16 longitudinally followed patients. **A**, Frequencies of CD25⁺ and CD25^{hi} cells. **B**, Relative proportion of Foxp3⁺CD25⁺ and Foxp3⁺CD25^{hi} populations. ** $P < .01$.

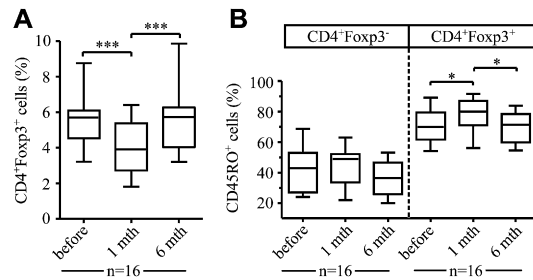


FIG E3. CD4⁺Foxp3⁺ Treg cells in 16 longitudinally followed patients. **A**, Significant decrease in CD4⁺Foxp3⁺ Treg cell numbers after 1 month of VIT. *** $P < .001$. **B**, CD45RO expression of CD4⁺Foxp3⁻ and CD4⁺Foxp3⁺ T cells. * $P < .05$.

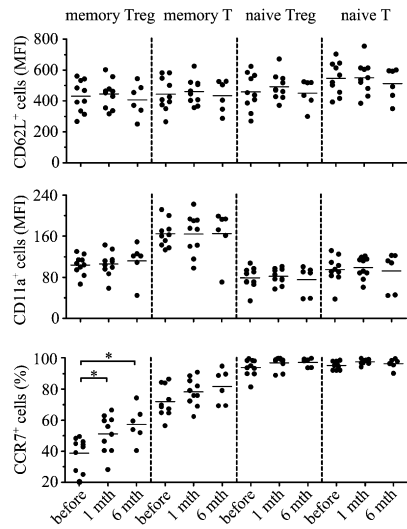


FIG E4. Expression of T-cell homing molecules in the course of VIT. CD62L, CD11a, and CCR7 expression of T-cell populations shown is indicated as *individual dots* and means (*horizontal line*). * $P < .05$ in 6 longitudinally followed patients. *MFI*, Mean fluorescence intensity.

TABLE E1. Characteristics of patients

No. of subjects	30
Mean $\pm$ SD age (y)	41.5 $\pm$ 12.7
Sex (M/F)	16/14
Anaphylaxis (grade*)	
I	0
II	26
III	4
IV	0
Threshold of positive IDT (μ g/mL; no. of subjects)	
<i>Vespula vulgaris</i>	0.001; 0
	0.01; 16
	0.1; 9
	1.0; 5
<i>Apis mellifera</i>	0.001; 0
	0.01; 0
	0.1; 1
	1; 1
Total IgE (kU/L $\pm$ SEM [<100])	88.4 $\pm$ 15.9
Specific IgE (kUA/L $\pm$ SEM [<0.35])	
<i>Vespula vulgaris</i>	9.5 $\pm$ 3.0
<i>Apis mellifera</i>	0.5 $\pm$ 0.13
Atopy	3/30

Brackets indicate reference values.

F, Female; IDT, intradermal test; M, male.

*According to the method described in Ring J, Messmer K. Incidence and severity of anaphylactoid reactions to colloid volume substitutes. Lancet 1977;1:466-9.