Immune Responses in Healthy and Allergic Individuals Are Characterized by a Fine Balance between Allergen–specific T Regulatory 1 and T Helper 2 Cells

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Abstract

The mechanisms by which immune responses to nonpathogenic environmental antigens lead to either allergy or nonharmful immunity are unknown. Single allergen-specific T cells constitute a very small fraction of the whole CD4$^+$ T cell repertoire and can be isolated from the peripheral blood of humans according to their cytokine profile. Freshly purified interferon-γ–, interleukin (IL)-4–, and IL-10–producing allergen-specific CD4$^+$ T cells display characteristics of T helper cell (Th)1-, Th2-, and T regulatory (Tr)1–like cells, respectively. Tr1 cells consistently represent the dominant subset specific for common environmental allergens in healthy individuals; in contrast, there is a high frequency of allergen-specific IL-4–secreting T cells in allergic individuals. Tr1 cells use multiple suppressive mechanisms, IL-10 and TGF-β as secreted cytokines, and cytotoxic T lymphocyte antigen 4 and programmed death 1 as surface molecules. Healthy and allergic individuals exhibit all three allergen-specific subsets in different proportions, indicating that a change in the dominant subset may lead to allergy development or recovery. Accordingly, blocking the suppressor activity of Tr1 cells or increasing Th2 cell frequency enhances allergen-specific Th2 cell activation ex vivo. These results indicate that the balance between allergen-specific Tr1 cells and Th2 cells may be decisive in the development of allergy.

Key words: peripheral tolerance • allergens • suppression • interleukins • immune regulation

Introduction

The immune system must distinguish between innocuous and pathological antigens to prevent unnecessary and self-destructive immune responses (1, 2). A central finding from experimental models and human studies shows that allergic diseases are due to an aberrant immune response mediated through a key effector cell, the Th type 2 cell and an associated cytokine pattern including IL-4, IL-5, and IL-13 (3, 4). Therefore, the most pronounced findings with potential relevance to allergy therapy are related directly to the control of these Th2 immune effectors. There is strong evidence that peripheral T cell regulation plays a crucial role in the control of harmful T cell responses. To avoid chronic cell activation and inflammation against nonpathogenic antigens through ingestion and inhalation, the immune system has developed efficient peripheral tolerance mechanisms. Since the early 1970s (5), different subtypes of regulatory and suppressor cells and mechanisms that may play a role in peripheral tolerance have been demonstrated, and their biology has been the subject of intensive investigation (6–14).

Abbreviations used in this paper: CTLA-4, CTL antigen 4; EF-1α, elongation factor-1α; L, ligand; PD-1, programmed death 1; PPD, purified protein derivative of *Mycobacterium bovis*; Tr, T regulatory.

A great deal of uncertainty remains about differentiation factors, antigen specificity, and mechanisms of action of T regulatory (Tr) cells. Several types of Tr cells have been described with a unique mechanism of action that varies depending on the experimental model. Recent studies have shown that Tr cells act as suppressor T cells that down-regulate other effector T cells and inflammation models in chronic infection, organ transplantation, and autoimmunity (10, 15–17). Studies on the immune response to allergens provide well-defined models for understanding the regulation and circumvention of antigen-specific T cell responses. The symptoms of IgE-mediated allergy rhinitis, conjunctivitis, and asthma can be ameliorated by the temporary suppression of mediators and immune cells (such as antihistamines and corticosteroids; reference 18). However, the only long-term solution for the treatment of allergy is allergen-specific immunotherapy by the administration of high doses of allergen or allergen peptides that specifically target T cells over a long period of time (19, 20). Successful venom and aeroallergen immunotherapy was found to be associated with the induction of peripheral tolerance in T cells by generation of Tr cells that secrete the suppressive cytokines IL-10 and TGF-β, suggesting that generation of Tr1 cells might play a role in healthy immune response (11, 21).

Studies on immune response to allergens in healthy individuals demonstrated that a peripheral T cell repertoire to allergens exists that recognizes the same T cell epitopes as allergic patients (22, 23). In the present work, we used a direct approach by purification, characterization, and frequency determination of allergen-activated T cells according to their cytokine secretion profile. This enabled a suitable human model to investigate how harmless environmental proteins are recognized and tolerated by the immune system. We showed that healthy and allergic immune response to common environmental proteins is characterized by a delicate balance in frequency of allergen-specific Tr1 cells and allergen-specific Th2 cells.

Materials and Methods

Study Population.   Heparinized peripheral blood samples from 31 healthy individuals (mean age, 36 yr) with no history of atopy and 8 birch pollen and 9 house dust mite allergic patients, who were diagnosed by positive skin test reactivity and high specific IgE antibodies (>0.70 kU/l, Pharmacia Cap assay) were studied. Total IgE levels of healthy individuals were <88 U/ml, and allergic individuals were between 92 and 298 U/ml. The study was approved by the ethical commission of Davos, Switzerland.

Antigens.   rBet v 1 of birch pollen (*Betula verrucosa*), rDer p 1 of house dust mite (*Dermatophagoides pteronyssinus*), rPyr c 5 of pear (*Pyrus communis*), and rCor a 1 of hazelnut (*Corylus avellana*) were used. rPyr c 5 and r Cor a 1 were provided by S. Vieths and D. Lüttkopf (Paul Ehrlich Institute, Langen, Germany). All of the allergens did not contain detectable amounts of LPS and were >99% pure. Purified protein derivative of *Mycobacterium bovis* (PPD) was obtained from the Serum Institute, Copenhagen, Denmark. Tetanus toxoid was obtained from the Institute Berne, Switzerland.

Purification of Allergen-specific IL-4–, IFN-γ–, and IL-10–secreting Cells.   PBMCs were isolated by Ficoll (Biochrom) density gradient centrifugation of peripheral venous blood, and cells were washed three times and resuspended in RPMI 1640 medium supplemented as described previously (8). 2.5×10^7 cells were stimulated with 0.3 μM antigens in 5 ml of medium in six-well plates in duplicates (Costar Corp.). After 12 h of stimulation in humidified 5% CO_2, cells were harvested and labeled with 50 μg/ml anti–IFN-γ/CD45, anti–IL-4/CD45, or anti–IL-10/CD45 Ab–Ab conjugates (Miltenyi Biotec) for 10 min at a concentration of 10^8 cells/ml in ice-cold RPMI 1640 medium (24). The cells were diluted with 37°C warm medium to a final concentration of 10^6 cells/ml and allowed to secrete and capture the respective cytokines for 45 min at 37°C. After capturing the secreted cytokines on their surface, cells were centrifuged at 300 *g* for 5 min at 4°C and resuspended at a concentration of 10^8 cells/ml in ice-cold buffer containing 0.5% BSA and 5 mM EDTA (both obtained from Sigma-Aldrich) in PBS. The cells were stained with 5 μg/ml PE-conjugated anti–IFN-γ, anti–IL-10, or anti–IL-4 for 10 min at 4°C. The cells were washed and resuspended in 400 μl BSA-EDTA PBS and magnetically labeled for 15 min at 4°C with 100 μl of anti-PE microbeads. After washing, labeled cells were purified by immunomagnetic separation (AutoMacs; Miltenyi Biotec). The cells were counterstained by FITC-labeled anti-CD4 and anti-CD8 mAb (Immunotech) and analyzed in a flow cytometer (Epics XL; Beckman Coulter; Fig. S1). The purity of allergen-specific $CD4^+$ cytokine-secreting cells was between 88 and 96%. The frequency of allergen-stimulated and unstimulated cells was calculated by dividing the number of purified cytokine-secreting $CD4^+$ T cells by the initial number of $CD4^+$ T cells. To obtain the frequency of allergen-specific, cytokine-secreting T cells, the unstimulated cytokine-secreting $CD4^+$ T cell frequency was subtracted from allergen-stimulated cell number.

T Cell Cultures.   The purified allergen-specific, cytokine-secreting T cells were used immediately or expanded in the presence of 1 nM doses of growth factors (IL-2 for IFN-γ–secreting T cells; IL-2 and IL-4 for IL-4-secreting T cells; and IL-2 and IL-15 for IL-10–secreting T cells; Novartis AG). Their cytokine profile was determined both immediately after purification and after in vitro expansion. Expanded IL-4–, IL-10–, and IFN-γ–producing cells were washed, and 5×10^5 cells were restimulated with a combination of mAbs to CD2, CD3, and CD28 molecules (each 0.5 μg/ml; CLB) in 500 μl supplemented RPMI 1640 medium in 48-well plates for 3 d in triplicates. Supernatants were harvested, and cytokines were determined by solid phase sandwich ELISAs for IFN-γ, IL-4, IL-5, IL-10, IL-13, and TGF-β as described previously (8, 21). Anti–IL-4 and anti–IFN-γ mAbs were provided by C.H. Heusser (Novartis Pharmaceuticals, Basel, Switzerland).

Allergen-specific T cell proliferative response was determined by stimulation of 2×10^5 PBMCs alone or together with expanded or freshly purified allergen-specific, cytokine-secreting T cells for 5 d with 0.3 μM of antigens in 200 μl of medium in 96-well flat-bottom tissue culture plates in triplicates (25). Autologous 3,000 rad irradiated PBMCs were used as APCs. Cells were pulsed with 1 μCi/well [³H]thymidine (Dupont and NEN Life Science Products), and incorporation of labeled nucleotide was determined after 8 h in an LKB β plate reader (Wallac and Amersham Biosciences). For polyclonal activation of T cells, plates were coated for 2 h at 37°C with 10 μg/ml anti-CD3. IL-10 was neutralized in cultures with 4 μg/ml anti-IL-10R mAb (provided by K. Moore, DNAX Research Institute, Palo Alto, CA; reference 26). TGF-β was neutralized in cultures with 100 ng/ml

of recombinant human soluble TGF-β receptor II/Fc chimeric protein (R&D Systems; reference 21). Programmed death 1 (PD-1) activity was neutralized in cultures with 5 μg/ml anti–human PD-1 (Bioscience Insight Biotechnology Ltd.). CTL antigen 4 (CTLA-4) activity was neutralized with 5 μg/ml anti-CD152 F(ab)² (Ancell and Qbiogene). The neutralizing activity of these approaches was controlled in titrated doses. Rabbit IgG, rat IgG, mouse IgG1, or BSA (Beckman Coulter) served as control.

Flow Cytometry, Immunohistology, and ELISPOT Assay. 2.5 × 10⁴ cells were stained with FITC-conjugated anti-CD25 mAb together with anti–CD4-ECD, anti–CD152-PE (CTLA-4; BD Biosciences), or anti–PD-1 (Bioscience Insight Biotechnology Ltd.) for 30 min at 4°C. Stained cells were fixed in 2% paraformaldehyde. The controls were FITC, PE, or ECD-conjugated mouse IgG1. For analysis of IL-10R, 5 × 10⁴ cells were stained with 50 μg/ml of anti–IL-10α mAb (DNAX Research Institute) for 30 min and washed with 2% FCS containing PBS. FITC-conjugated anti–rat Ig was the second Ab used for 30 min.

Intracellular cytokines were detected after anti-CD2, anti-CD3, and anti-CD28 mAb stimulation for 12 h. 2 μM monensin (Sigma-Aldrich) was added during the last 10 h (11). Intracytoplasmic cytokine profile of in vitro–expanded allergen-specific T cells was determined as aforementioned. Labeled mAbs for cytokines were obtained from BD Biosciences. Immune histology was performed in cytospins of freshly purified cells by using anti–human TGF-βRI, anti–human TGF-βRII, or rabbit IgG as isotype control (all obtained from Santa Cruz Biotechnology Inc.) as described previously (27).

10⁶ cells/ml PBMCs from six healthy donors were stimulated in 200 μl of medium 96-well flat bottom ELISPOT plates for 18 h (Euroclone Ltd.). Locally produced IL-4, IFN-γ, and IL-10 were captured by specific mAb. After cell lysis, trapped cytokine molecules were revealed by a secondary biotinylated detection antibody, which is in turn recognized by streptavidin conjugated to alkaline phosphatase. Colored "purple" spots developed after substrate addition were determined (ImmunoSpot; Cellular Technology Ltd.). The number of spots determined in triplicates of unstimulated wells was subtracted from 0.3 μM Der p 1–stimulated wells. 18 h was found to be the optimal time for the determination of frequency of cytokine-secreting cells, as it is the time point for highest cytokine secretion before T cell proliferation starts.

Quantitative Real-Time PCR. Immediately after purification, antigen-specific, cytokine-secreting T cells were lysed with RNeasy lysis buffer, and the RNA was isolated using the RNeasy mini kit (QIAGEN) and eluted in 30 μl ddH₂O. Reverse transcription was performed with TaqMan® reverse transcription reagents with random hexamers (Applied Biosystems). The PCR primers and probes detecting elongation factor-1α (EF-1α), IL-10, TGF-β, IL-13, and IFN-γ were designed based on sequences reported in GenBank. Primers used were as follows: EF-1α forward primer, 5′-CTGAACCATCCAGGCCAAAT-3′, EF-1α reverse primer, 5′-GCCGTGTGGCAATCCAAT3′; IL-13 forward primer A, 5′-GCCCTGGAATCCCTGATCA-3′, IL-13 reverse primer A, 5′-GCTCAGCATCCTCTGGGTCTT-3′; IFN-γ forward primer B, 5′-TCTCGGAAACGATGAAAT-ATACAAGTTAT-3′, IFN-γ reverse primer B, 5′-GTAACA-GCCAAGAGAACCCAAAA-3′; IL-10 forward primer, 5′-GGCGCTGTCATCGATTTCTT-3′, IL-10 reverse primer, 5′-TTGGAGCTTATTAAAGGCATTCTTC-3′; TGF-β1 forward primer, 5′-AAATTGAGGGCTTTCGCCTTA-3′, and TGF-β1 reverse primer, 5′-GAACCCGTTGATGTCCACTTG-3′. cDNAs were amplified using SYBR®-PCR mastermix (Applied Biosystems) according to the recommendations of the manufacturer in a total volume of 25 μl in a sequence detection system (ABI
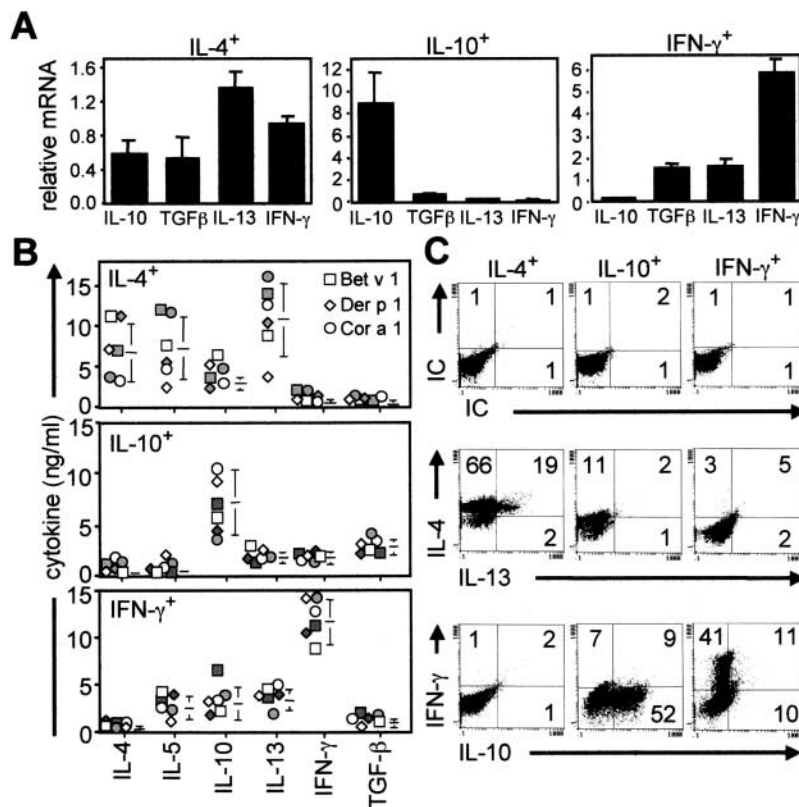

Figure 1. Allergen-specific IL-4–, IFN-γ–, and IL-10–secreting T cells represent Th2-, Th1-, and Tr1-like cells. (A) IL-10, IL-13, IFN-γ, and TGF-β mRNA were quantified by real-time PCR immediately after isolation of Der p 1– or Bet v 1–specific, cytokine-secreting T cells and their relative expression compared with the housekeeping gene EF-1α. The same results were obtained in three independent experiments. (B) Bet v 1–, Der p 1–, and Cor a 1–specific IL-4–, IFN-γ–, and IL-10–secreting T cells (one allergic and one healthy donor each; closed symbols, allergic donors; open symbols, healthy donors) were in vitro expanded for 2 wk, and their cytokine profile was determined in supernatants by ELISA 72 h after anti-CD2, anti-CD3, and anti-CD28 mAb stimulation. (C) Intracytoplasmic cytokine profile of in vitro–expanded allergen-specific T cells (the same results were obtained in 10 additional experiments). Percentage of positive cells is shown in each quadrant.
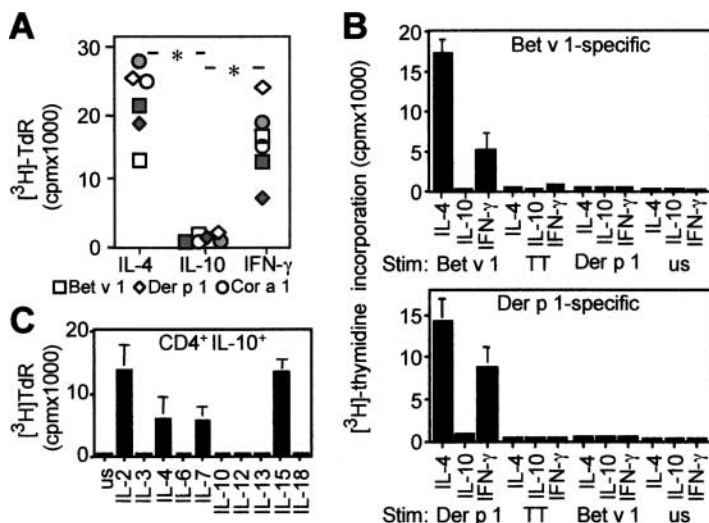
Figure 2. Specificity and growth requirements of purified allergen-specific T cells. (A) Bet v 1–, Der p 1–, and Cor a 1–specific IL-4–, IFN-γ–, and IL-10–secreting T cells (one allergic and one healthy donor each; closed symbols, allergic donors; open symbols, healthy donors) were purified and in vitro expanded, and 5×10^5 cells were cocultured with 5×10^5 autologous irradiated PBMCs as an APC source in the presence of the same antigen. [³H]Thymidine incorporation (TdR) was determined after 5 d. *, P < 0.001. (B) Purified Bet v 1–specific and Der p 1–specific T cells did not show any proliferative response to different control antigens (two representative of six experiments are shown; mean ± SD of triplicate cultures). TT, tetanus toxoid; us, unstimulated. (C) Growth factor requirements of antigen-specific IL-10–secreting T cells. After purification, Bet v 1– or Der p 1–specific IL-10–secreting T cells were first expanded for 10 d in the presence of IL-2, washed, and cultured (5×10^4 cells) with 1-nM doses of different cytokines. [³H]Thymidine incorporation was determined after 3 d (mean ± SD of three independent experiments are shown).

PRISM 7000; Applied Biosystems). Relative quantification was performed as described previously (28). All amplifications were performed in duplicates.

Statistical Interpretation. Data are expressed as mean ± SEM. Student's *t* test, Z test, and Mann–Whitney U test were used for statistical analysis.

Online Supplemental Material. Fig. S1 contains sample data of the purification and frequency calculation of allergen-specific CD4⁺ cells according to their cytokine profile. Details of flow cytometric analysis are shown, from allergen stimulation to frequency calculation of purified IL-10–secreting Tr1 cells. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20032058/DC1.

Results

Allergen-specific IL-4–, IFN-γ–, and IL-10–secreting T Cells Represent Th2-, Th1-, and Tr1-like Cells. To investigate the regulation of specific immune response against environmental protein antigens, we isolated CD4⁺ T cells specific to several food and aeroallergens from healthy and allergic individuals according to their IL-4, IFN-γ, and IL-10 secretion profile (Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20032058/DC1). To confirm their cytokine profile, mRNA of IL-10, IL-13, IFN-γ, and TGF-β were quantified immediately after isolation (Fig. 1 A). Relative to the housekeeping gene EF-1α, IL-10–secreting T cells expressed significantly high IL-10 mRNA and IFN-γ–secreting T cells expressed significantly high IFN-γ mRNA. IL-13 mRNA was dominant in IL-4–secreting T cells. Quantification of cytokines after in vitro expansion of IL-4–, IL-10–, and IFN-γ–secreting T cells revealed that these subsets contain Th2-like (IL-4, IL-5, and IL-13 high), Th1-like (IFN-γ high), and Tr1-like (IL-10 and TGF-β high) cells, respectively (all P < 0.0001 compared with other subsets; Fig. 1, B and C). In addition, all three purified subsets consisted of some Th0 cells, which secrete both Th1 and Th2 cytokines as well as IL-10 and TGF-β. There was no difference in cytokine profile at the single T cell subset level between allergic and healthy individuals.

The antigen specificity of purified cytokine-secreting T cells was studied by stimulation with the allergen that was originally used for stimulation before purification and several control antigens in the presence of autologous APCs. Consistently, IL-10–secreting T cells showed very little or no allergen-induced proliferation (Fig. 2 A). The proliferative response of allergen-specific IL-4– and IFN-γ–secreting T cells was significantly high compared with IL-10–
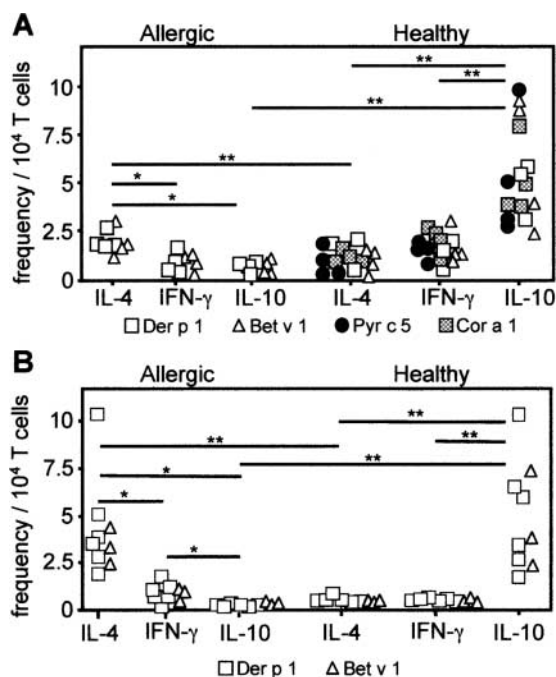

Figure 3. Increased frequency of allergen-specific Tr1 cells in healthy individuals and Th2 cells in allergic individuals. (A) Frequency of Der p 1– and Bet v 1–specific, cytokine-secreting CD4⁺ T cells from eight allergic individuals and Cor a 1–, Bet v 1–, Der p 1–, and Pyr c 5–specific, cytokine-secreting CD4⁺ T cells from 15 healthy individuals out of pollen season. (B) Frequency of Der p 1–specific T cells in six allergic and six healthy, and Bet v 1–specific T cells in three allergic and three healthy individuals by ELISPOT assay. *, P < 0.01; **, P < 0.001.

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secreting T cells. There was no difference between different allergens. T cells purified by certain antigen stimulation did not show any cross-reactivity against control antigens (Fig. 2 B). All three subsets purified by Bet v 1 stimulation responded to Bet v 1, but not to tetanus toxoid and Der p 1. Similarly, Der p 1–specific T cell subsets showed proliferative response to Der p 1, but not to tetanus toxoid and Bet v 1 as control antigens. Although they did not proliferate by antigen stimulation, IL-10–secreting T cells used IL-2, IL-4, IL-7, and IL-15 as growth factors and showed significant proliferation (Fig. 2 C). Together with the quantitative cytokine mRNA profiles of freshly purified cells, these data demonstrate that allergen-specific Tr1-, Th1-, and Th2-like cells can be purified from human peripheral blood.

Balance between Allergen-specific Tr1 Cells and Th2 Cells Characterizes Healthy and Allergic Immune Response. As it was possible to purify single allergen-specific Th1-, Th2-, and Tr1-like cells, their frequency and functional properties were investigated in the next step. The frequency of T cell subsets specific to different mucosal allergens was compared in healthy and allergic individuals using two different techniques. Recombinant major allergens of house dust mite (Der p 1) and birch pollen (Bet v 1) were used as aeroallergens, and pear (Pyr c 5) and hazelnut (Cor a 1) were used as food antigens to analyze the frequency of spe-

cific Th1-, Th2-, and Tr1-like cells. Although specific T cells that belong to all three subsets were detectable in both healthy and allergic individuals, allergen-specific IL-10–secreting T cells were the predominant subset in healthy individuals. We found similar results for each allergen. In contrast, an increased frequency of IL-4–secreting T cells was observed in allergic patients (Fig. 3 A). ELISPOT was used as an alternative method in allergen-stimulated PBMC cultures and demonstrated a similar frequency distribution of Der p 1– and Bet v 1–specific T cell subsets (Fig. 3 B).

As aforementioned, all three subsets of single allergen-specific T cells are present in both healthy and allergic individuals. Accordingly, their role on allergen-induced T cell proliferation and whether this is influenced by changing their ratios was investigated. We assayed the allergen-induced proliferation of IL-4– and IL-10–secreting T cells by adding those purified cells back into autologous PBMCs. First, the antigen-specific suppressor effect of IL-10–secreting T cells was analyzed (Fig. 4 A). Bet v 1– and Der p 1–specific IL-4– and IL-10–secreting T cells were separately purified from the same healthy individuals. Their frequency was increased up to 10 times higher than initial levels in PBMCs. PBMCs alone did not show allergen-induced T cell proliferation, which was achieved by increasing the numbers of allergen-specific IL-4–secreting T cells. Der p 1–specific IL-10–
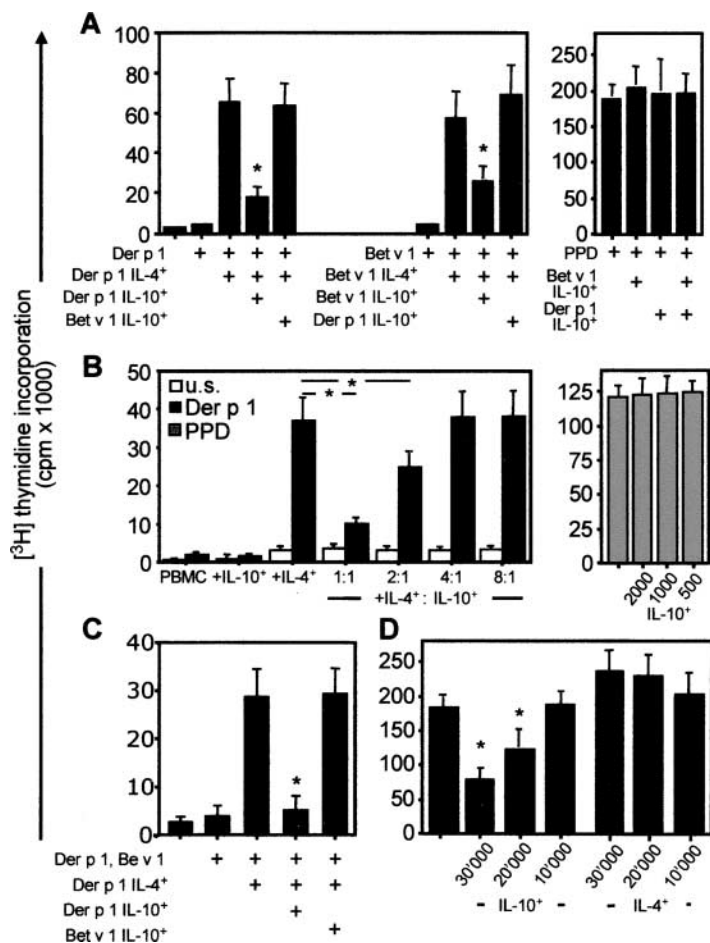

Figure 4. Antigen-specific suppression by Tr1 cells. (A) Der p 1–specific and Bet v 1–specific IL-4–secreting and IL-10–secreting T cells were purified from healthy individuals. Their frequency was calculated in CD4+ T cells, and 2×10^5 PBMCs were immediately reconstituted by increasing their frequency by 10 times (IL-4–secreting T cells, 0.02–0.2%; IL-10–secreting T cells, 0.05–0.5%). Cells were stimulated with the respective antigens and PPD. (B) Der p 1–specific IL-4–secreting and IL-10–secreting T cells were purified and in vitro expanded by IL-2/IL-4 and IL-2/IL-15, respectively. 2×10^5 PBMCs were enriched with 1,000 Der p 1–specific IL-4– or IL-10–secreting T cells or their combinations in IL-4–secreting/IL-10–secreting T cell ratios 1,000/1,000 (1:1), 1,000/500 (2:1), 1,000:250 (4:1), and 1,000:125 (8:1). Cells were stimulated with 0.3 μM Der p 1 or 1 μg/ml PPD. Der p 1–specific, IL-10–secreting T cells added to PPD-stimulated PBMC cultures at indicated numbers did not show any suppression. (C) The same experimental design as in A was used, and cells were stimulated with both Der p 1 and Bet v 1 (0.3 μM each). (A–C) [³H]Thymidine incorporation was determined after 5 d. The same results were obtained in three other experiments. *, P < 0.001. (D) 10^5 PBMCs were stimulated with anti-CD3 in the presence of different amounts of IL-10–secreting and IL-4-secreting T cells. [³H]Thymidine incorporation was determined after 3 d. Data represent two different experiments. *, P < 0.01.

secreting T cells only suppressed Der p 1–stimulated, but not Bet v 1– or PPD-stimulated proliferation. Similarly, Bet v 1–specific IL-10–secreting T cells only suppressed Bet v 1–stimulated, but not Der p 1– or PPD-stimulated proliferation. There was no cross-suppression of Der p 1–specific IL-10–secreting cells on Bet v 1 stimulation and Bet v 1–specific IL-10–secreting T cells on Der p 1 stimulation, as well as both IL-10–secreting T cells on PPD stimulation.

The retention of suppressive activity of Tr1 cells after expansion ex vivo is obviously an absolute prerequisite for usage in a possible cellular therapeutic approach. We tested whether in vitro–expanded IL-10–secreting T cells maintain their suppressive activity. PBMCs of healthy individuals showed no or very limited proliferative response to Der p 1. The addition of in vitro–expanded Der p 1–specific IL-4, but not IL-10–secreting T cells significantly enhanced Der p 1–induced proliferation (Fig. 4 B). Increased numbers of in vitro–expanded IL-10–secreting T cells significantly suppressed IL-4–secreting T cell–mediated proliferation in Der p 1–stimulated PBMCs. A 1:1 ratio of effector (IL-4–secreting) to suppressor (IL-10–secreting) T cells was required for full suppression of T cell proliferation. In contrast, PPD-stimulated proliferation was not suppressed by Der p 1–specific IL-10–secreting T cells.

To test whether the IL-10–secreting T cells induce bystander suppression on other T cells in their vicinity, we cultured Der p 1–specific, IL-4–secreting cells with freshly purified Der p 1– or Bet v 1–specific IL-10–secreting cells in the presence of both Der p 1 and Bet v 1 (Fig. 4 C). Der p 1–specific, IL-4–secreting T cell proliferation was suppressed by Der p 1–, but not Bet v 1–specific Tr1 cells. As few as 1,000 Tr1 cells were able to induce antigen-specific suppression in 200,000 PBMCs. High numbers of freshly purified Tr1 cells (at least 20,000 in 100,000 PBMCs) re-

sulted in a nonspecific suppressive activity on anti-CD3–stimulated T cells (Fig. 4 D). In comparison, IL-4–secreting T cells did not exert any suppressive effect on anti-CD3 stimulation. These data demonstrate allergen-specific suppressor activity of Tr1 cells and the importance of the balance between allergen-specific Th2 and Tr1 cells for allergen-induced T cell activation.

Allergen-specific Tr1 Cells Use Multiple Suppressor Factors. Molecules that may play a role on suppressive mechanisms of allergen-specific IL-10–secreting T cells were analyzed
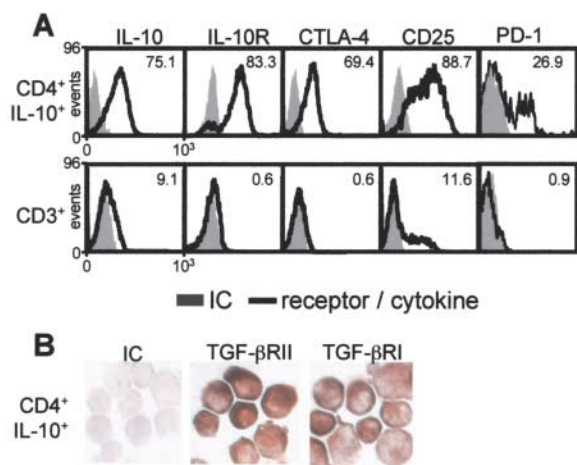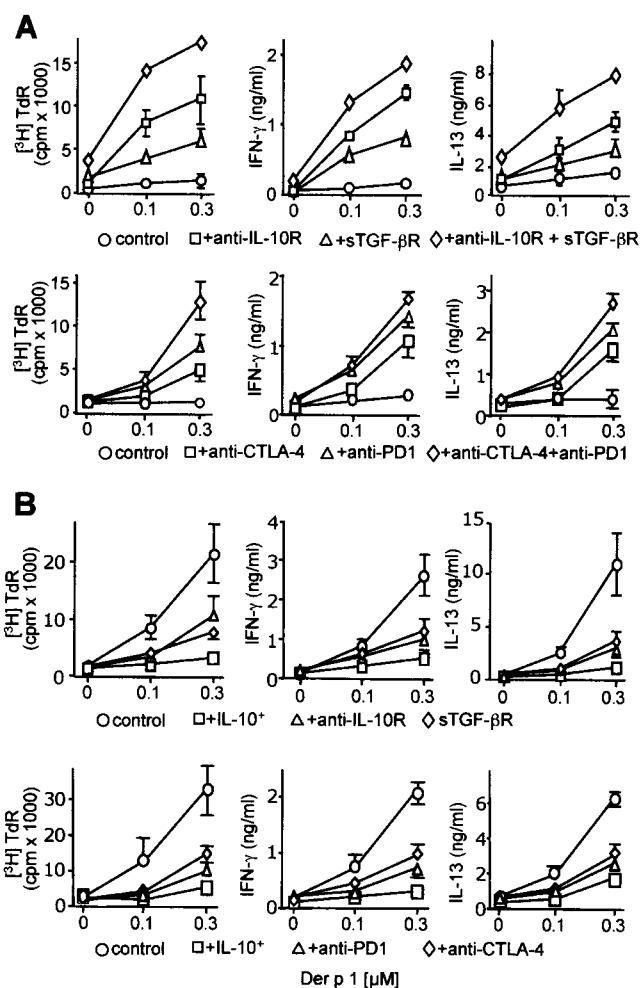

Figure 6. Multiple suppressive mechanisms play a role in peripheral allergen tolerance. (A) Endogenous IL-10, TGF-β, or both as well as CTLA-4, PD-1, or both were neutralized in Der p 1–stimulated PBMCs of healthy individuals. [³H]Thymidine incorporation (TdR), IFN-γ, and IL-13 were determined at day 5. (B) Der p 1–specific proliferation of PBMCs from house dust mite allergic patients was suppressed by 10 times increased frequency of Der p 1–specific, IL-10–secreting T cells (IL-10⁺). The activity of IL-10, TGF-β, CTLA-4, and PD-1 were neutralized. [³H]Thymidine incorporation (TdR), IFN-γ, and IL-13 were determined at day 5. (control) Isotype control antibody. (A and B) Same results were obtained in four independent experiments (two Bet v1 and two Der p 1) all performed with freshly purified cells without in vitro expansion. Bet v 1 stimulation in birch pollen–allergic individuals gave similar results. All of the neutralization experiments were significantly different from the control and IL-10–secreting T cell–suppressed condition at 0.3 μM allergen doses. P < 0.001.

Figure 5. Expression of suppressor molecules on IL-10–secreting T cells. (A) Immediately after purification, antigen-specific IL-10–secreting T cells of healthy individuals were analyzed for intracytoplasmic IL-10, surface IL-10R, CTLA-4, CD25, and PD-1 by flow cytometry. Data are compared with CD3⁺ T cells of the same donor stimulated with the same antigen. (B) TGF-β RI and RII expression by immunohistology. (A and B) Same results were obtained in three independent experiments.

on freshly purified cells (Fig. 5). IL-10–secreting T cells expressed high amounts of IL-10, IL-10Rα chain, TGF-β receptors I and II, CTLA-4, CD25, and PD-1, suggesting that multiple suppressor factors may play a role on suppression of allergen-specific Th2 cells by Tr1 cells in healthy individuals.

Neutralization experiments revealed that all four suppressive mechanisms may play a role in suppression of allergen-specific Th2 cells. Der p 1– or Bet v 1–stimulated PBMCs of healthy individuals did not show any T cell proliferation. In both cases, neutralization of IL-10 and TGF-β activity significantly enhanced antigen-induced proliferation as well as IL-13 and IFN-γ production (Fig. 6 A). In addition, two other mechanisms apparently function in healthy immune response to allergens because neutralization of CTLA-4 or PD-1 significantly enhanced T cell proliferation and IFN-γ and IL-13 secretion in healthy individuals. In allergic individuals, Der p 1–induced T cell proliferation was significantly high in PBMCs of house dust mite allergic donors. A very clear suppression was achieved by increasing the frequency of Der p 1–specific, IL-10–secreting T cells. This suppression was partially inhibited by blocking of IL-10R, TGF-β, CTLA-4, or PD-1 (Fig. 6 B). In these experiments, IL-10R–blocking mAbs may block IL-10R on the APCs as well as on Tr1 cells; in addition, TGF-β secreted from both Tr1 cells and APCs can be neutralized by sTGF-βR. CTLA-4 and PD-1 are blocked particularly on the surface of Tr1 cells. These data demonstrate that IL-10, TGF-β, CTLA-4, and PD-1 cooperate in the suppression of immune response to allergens.

Discussion

The present work demonstrates that immune response to allergens in health and disease is the result of a balance between allergen-specific Tr1 cells and allergen-specific Th2 cells. Active regulation has emerged as a very essential mechanism for both inducing and equally importantly maintaining specific immunological nonresponsiveness. By analyzing Tr1 cells specific to various food or inhalant antigens, we demonstrated that similar mechanisms take place in healthy immune response to mucosal allergens. The IL-10–secreting allergen-specific T cells represented the predominant subset with significantly high frequency in comparison to IL-4– and IFN-γ–secreting T cells in healthy individuals. Both ELISPOT for cytokines and purification of cytokine-secreting T cells gave similar frequency numbers. Mechanisms that control the in vivo shift between allergen-specific T cell subsets remain to be elucidated. It can be hypothesized that a switch in cytokine profile and apoptosis of effector T cells may play a role in the generation of an atopic phenotype (29, 30). The stability of cytokine profile in differentiated effector and memory T cell subsets in humans is not fully known, and recent studies have demonstrated that lineage-committed memory T cell subsets are responsive to cytokine signals of the opposing lineage (30, 31). In addition, Tr1 cells do not appear to be anergic and may efficiently expand in vivo (32) as they are shown to proliferate by IL-2, IL-4, IL-7, and IL-15 in the present work.

There is clear evidence from various animal models and human studies for an active mechanism of immune suppression, whereby a distinct subset of T cells inhibits the activation of conventional T cells in the periphery (7, 13, 14, 33). This Tr cell population has been determined as CD4+CD25+ T cells. They can prevent the development of autoimmunity, indicating that the normal immune system contains a population of professional regulatory T cells. Elimination of CD4+CD25+ T cells leads to spontaneous development of various autoimmune diseases, such as gastritis or thyroiditis, in genetically susceptible hosts. The frequency of CD4+CD25+ Tr cells is ∼10–15% of CD4+ T cells, whereas the frequency of IL-10–secreting T cells of a single allergen specificity ranges between 0.1 and 0.007% of CD4+ T cells. This shows that the frequency of single allergen-specific Tr1 cells, which are also CD4+ CD25+, ranges between 1 in 1,000 and 1 in 20,000 of the whole CD4+ CD25+ Tr cell population.

Although many aspects of the mechanisms by which suppressor cells exert their effects remain to be elucidated, it is well established that Tr cells suppress immune responses via cell-to-cell interactions and/or the production of IL-10 and TGF-β (10, 11, 34, 35). Tr1 cells specific for a variety of antigens arise in vivo, but may also differentiate from naive CD4+ T cells in the presence of IL-10 in vitro (36). The nonspecific T cell suppressor activity of IL-10 and TGF-β has been consistently reported in experiments with high amounts of exogenously added suppressor cytokines (21, 25). However, the present work demonstrates that Tr1 cells display antigen-specific suppressor activity in very low numbers. If the number of cells exceed a threshold that provides sufficient quantities of suppressor signals, apparently they show nonspecific suppression. Depending on their frequency, the first T cell that contacts the APC may be very critical in the subsequent decision to stimulate or suppress the specific immune response. If the first T cell to contact the APC is a Tr1 cell, it may silence or regulate the maturation of APC. IL-10 down-regulates the antigen-presenting capacity, such as HLA-DR expression, costimulatory molecules, and several cytokines in dendritic cells and monocytes/macrophages (37). Recently, differentiation of a distinct dendritic cell subset in the presence of IL-10 has been demonstrated that induces tolerance through the generation of Tr1 cells (38). In addition, exposure of mature pulmonary dendritic cells to respiratory allergens stimulated the development of Tr1-like cells, which was dependent on dendritic cell IL-10 production (39). Together, these findings suggest that IL-10–secreting T cells may regulate the functional state of APCs in a way that these APCs can now promote the generation of Tr1 cells.

CD4+CD25+ T cells are the only lymphocyte subpopulation in mice and humans that express CTLA-4 constitutively. The expression apparently correlates with the suppressor function of CTLA-4. As demonstrated in the present work, the blocking of CTLA-4 activity of Tr1 cells

reverses suppression in cocultures of CD4$^+$CD25$^+$ and CD4$^+$CD25$^-$ T cells (40). Similarly, the treatment of mice that were recipients of CD4$^+$CD45RB^{low} T cells with CTLA-4–blocking agents, abrogated the suppression of inflammatory bowel disease (41). These studies indicate that signals that result from the engagement of CTLA-4 by its ligands, CD80 or CD86, are required for the induction of suppressor activity. Under some circumstances, the engagement of CTLA-4 on the CD4$^+$CD25$^+$ T cells by antibody or by CD80/CD86 might lead to inhibition of the TCR-derived signals that are required for the induction of suppressor activity.

A subset of human Tr1 cells expressed functional PD-1 in the present work. PD-1 is an immunoreceptor tyrosine-based inhibitory motif–containing receptor expressed upon T cell activation. PD-1–deleted mice develop autoimmune diseases, suggesting an inhibitory role for PD-1 in immune responses (42). Members of the B7 family, PD-ligand (L)1 and PD-L2, are ligands for PD-1. PD-1/PD-L engagement on murine CD4 and CD8 T cells results in inhibition of proliferation and cytokine production. T cells stimulated with anti-CD3/PD-L1Fc-coated beads display dramatically decreased proliferation and IL-2 production (43).

Collectively, our results indicate that the control of Th2 immune response against naturally exposed harmless environmental antigens is mediated by Tr1 cells in humans. Effector (allergen-specific Th2) and suppressor (allergen-specific Tr1) T cells exist in both healthy and allergic individuals in certain amounts. Their ratio determines the development of a healthy or an allergic immune response. These data may explain the spontaneous development and spontaneous healing of allergic diseases. Although in low frequency, the existence of potential suppressive allergen-specific Tr1 cells in allergic individuals suggests a possible way of treatment. The knowledge of this cellular and molecular basis is pivotal in understanding the mechanisms of immune tolerance or allergy development against harmless environmental proteins.

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