

Induction and maintenance of allergen-specific FOXP3⁺ Treg cells in human tonsils as potential first-line organs of oral tolerance

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Background: Tonsils are strategically located in the gateway of both alimentary and respiratory tracts representing the first contact point of food and aeroallergens with the immune system. Tonsillectomy removes only the palatine tonsils and sometimes adenoids. Lingual tonsil is anatomically big and remains lifelong intact.

Objective: The aim of this study was to demonstrate cellular and molecular mechanisms of oral tolerance induction to food and aeroallergens in human tonsils.

Methods: Tonsil allergen-specific FOXP3⁺ regulatory T (Treg) cells, plasmacytoid dendritic cells (pDCs), and myeloid dendritic cells were characterized by flow cytometry and suppressive assays. Intracellular staining, [³H]-thymidine incorporation, and carboxy-fluorescein succinimidyl ester dilution experiments were performed. Tonsil biopsies were analyzed by confocal microscopy.

Results: CD4⁺FOXP3⁺ Treg cells and pDCs constitute important T- and dendritic cell-compartments in palatine and

lingual tonsils. Tonsil pDCs have the ability to generate functional CD4⁺CD25⁺CD127⁺FOXP3⁺ Treg cells with suppressive property from naive T cells. CD4⁺FOXP3⁺ Treg cells proliferate and colocalize with pDCs *in vivo* in T-cell areas of lingual and palatine tonsils. Tonsil T cells did not proliferate to common food and aeroallergens. Depletion of FOXP3⁺ Treg cells enables the allergen-induced proliferation of tonsil T cells, indicating an active role of Treg cells in allergen-specific T-cell unresponsiveness. High numbers of major birch pollen allergen, Bet v 1-specific CD4⁺FOXP3⁺ Treg cells, are identified in human tonsils compared with peripheral blood. A positive correlation between the percentages of FOXP3⁺ Treg cells and pDCs is observed in tonsils from nonatopic individuals. **Conclusion:** Functional allergen-specific Treg cells are identified both in lingual and in palatine tonsils. (J Allergy Clin Immunol 2011;■■■:■■■-■■■.)

Key words: Immune tolerance, tonsils, allergen-specific Treg cells, plasmacytoid dendritic cells, myeloid dendritic cells

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The authors' laboratories are supported by Swiss National Foundation grants 32-125249 and 320030-132899 and Christine Kühne-Center for Allergy Research and Education (CK-CARE). O.P. was supported by a postdoctoral fellowship from Ministerio de Educación y Ciencia and FECYT (Spain) and W.W.K. by NIH contract HHSN272200700046C.

Disclosure of potential conflict of interest: M. Akdis has received research support from the Swiss National Foundation, European Commission FW7 (McDALL), and Predicta. C. A. Akdis has received research support from Novartis, Stallergenes, the Swiss National Science Foundation, Global Allergy and Asthma European Network, and Christine Kühne-Center for Allergy Research and Education; is a Fellow and interest group member of the American Academy of Allergy, Asthma & Immunology; is Vice President of the European Academy of Allergy and Clinical Immunology; and was a committee member for GA²LEN. The rest of the authors declare that they have no relevant conflicts of interest.

Received for publication March 11, 2011; revised September 20, 2011; accepted for publication September 28, 2011.

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0091-6749/\$36.00

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doi:10.1016/j.jaci.2011.09.031

Tonsils are secondary lymphoid organs located in the oropharynx and the nasopharynx that constitute part of the mucosa-associated lymphoid tissue.¹ There are 4 areas of tonsil tissue: the paired palatine tonsils (at both sides in the back of the mouth), the nasopharyngeal or adenoid tonsil (attached to the roof of the pharynx), the paired tubal tonsils (at the openings of the Eustachian tubes), and the lingual tonsil (located at the back of the tongue).² Palatine tonsils and sometimes adenoids are removed by tonsillectomy without disturbing the integrity of the relatively big lingual tonsil as well as tubal tonsils. The percentage of children and young adults who undergo tonsillectomy has been significantly reduced over the past years, and it is still controversial whether the operation is always beneficial for the patient.³ Tonsils constitute the first contact point of the immune system to food or aeroallergens before their degradation by digestive enzymes and highly acidic gastric secretions.⁴ During inhalation, particulate aeroallergens reach to a speed of around 100 km/h and hit the surface of adenoids. Similarly, swallowing of food particles squeezes them between the oropharynx wall and the lingual tonsil. In addition, the highly cryptic surface structure enables long-term contact with food and aeroallergens. Although there is a strong rationale due to their anatomical location and histology, the potential role of tonsils in oral tolerance induction has not been elucidated so far.

Abbreviations used

CFSE: Carboxy-fluorescein succinimidyl ester
 DCs: Dendritic cells
 mDCs: Myeloid dendritic cells
 PC5: Phycoerythrin and cyanin 5
 pDCs: Plasmacytoid dendritic cells
 TDCs: Tonsil dendritic cells
 TLR: Toll-like receptor
 TMCs: Tonsil mononuclear cells
 Treg: Regulatory T

Studies on immune response to allergens in healthy individuals demonstrated that a peripheral T-cell repertoire to allergens exists,^{5,6} and accordingly, active regulation emerges as a very essential mechanism for inducing and maintaining peripheral tolerance to allergens.⁷ The generation of regulatory T (Treg) cells constitutes a main component of oral tolerance induction that is favored under certain circumstances and in particular microenvironments.^{8–12} Broadly, T cells with immunoregulatory capacity can be classified into 2 groups: the thymus-derived naturally occurring CD4⁺CD25⁺FOXP3⁺ Treg cells¹³ and the inducible Treg cells generated outside the thymus after antigenic stimulation: (i) inducible FOXP3⁺ T cells,^{14,15} (ii) IL-10-producing Treg1 cells,^{16,17} and (iii) TGF- β -producing T_H3 cells.¹⁸ Several previous studies described different FOXP3⁺ Treg-cell populations in palatine tonsils,^{19,20} but the detailed phenotypic and functional properties of purified tonsil Treg cells or whether allergen-specific FOXP3⁺ Treg cells can be found in human tonsils remained largely unknown.

Dendritic cells (DCs) play a pivotal role in the orchestration of immune responses.^{21–24} In humans, circulating DCs can be broadly divided into 2 groups: (i) myeloid dendritic cells (mDCs) and (ii) plasmacytoid dendritic cells (pDCs).²⁵ Both subsets express a different repertoire of Toll-like receptors (TLRs) and display a diverse cytokine signature after microbial stimulation.²⁶ mDCs selectively express TLR2-6 and -8 and respond to bacterial and viral infections by producing large amounts of IL-12. In contrast, pDCs constitutively express the endosome-associated TLR7 and -9 and they are the main producers of type I interferons in humans.^{27,28} Although several functional studies using tonsil DCs have been reported,^{29–33} little is known about how tonsil DC subsets drive innate and adaptive immune responses.

In this study, we investigated whether allergen tolerance induction through the generation of Treg cells might occur in human tonsils. Most of the data represent the *in vivo* situation in humans with direct analysis of the cells and tissues. Allergen-specific CD4⁺FOXP3⁺ Treg cells with suppressive activity exist in human palatine and lingual tonsils, indicating an active role of tonsils as the first step of oral tolerance.

METHODS**Tonsil samples**

This study has been reviewed and approved by the ethic committee of Cantons of Graubünden and Zurich. Human palatine tonsils were obtained from the hospitals of Davos and Chur, Switzerland. Noninflamed tonsils from patients undergoing tonsillectomy were included in the study. Tonsil samples from atopic donors were compared with those from healthy individuals. Atopic patients showed a polyallergic status according to self-reported allergies to different pollen and foods. All atopic individuals

included in this study displayed specific IgE to Bet v 1 or Phl p 1a or Phl p 5a or Cor a 1, as determined by ELISA. Nonatopic individuals did not show specific IgE to any of the common allergens tested. Human palatine tonsil tissues from Satakunta Central Hospital, Pori, Finland, were used for the initial mRNA expression screening. Lingual tonsil biopsies were performed in 3 patients recruited from the Zagreb School of Medicine, Croatia, with the diagnosis of hypertrophic lingual tonsil, metastatic cervical lymph node, and inflamed cyst of epiglottis. Lingual tonsil tissues were intact and not infiltrated with tumor cells or cysts in any of the patients.

Tonsil mononuclear cell isolation and purification of tonsil CD4⁺FOXP3⁺ Treg cells

Single-cell suspensions of tonsil mononuclear cells (TMCs) were isolated immediately after surgery by mechanical disruption. The tissues were chopped and grounded with medium through a 40- μ M mesh. After soft-lysis and several washing steps with medium, the cells were purified by centrifugation on a density gradient (Bicoll; Biochrom, Berlin, Germany). Tonsil dendritic cells (TDCs) were isolated from TMCs by negative selection by using antibodies against CD3, CD19, CD16, CD14, and CD326 coupled to microbeads in the presence of fragment crystallizable receptor (FcR)-blocking reagent and the autoMACS magnetic separation system (Miltenyi Biotec, Bergisch Gladbach, Germany). Tonsil and circulating CD4⁺FOXP3⁺ Treg cells were purified from TMCs by using the CD4⁺CD25⁺ Regulatory T Cell Isolation Kit (Miltenyi Biotec) following the manufacturer's indications (see Fig E1 in this article's Online Repository at www.jacionline.org).

PBMC isolation and naive CD4⁺ T-cell and peripheral blood CD4⁺FOXP3⁺ Treg-cell purification

Human PBMCs were isolated from heparinized whole blood or buffy coats from healthy donors by centrifugation on a density gradient. Naive CD4⁺ T cells were purified by using the Naive CD4⁺ T Cell Isolation Kit (Miltenyi Biotec). The obtained negative fraction was further purified with anti-CD8, anti-CD14, and anti-CD45RO coupled to microbeads (Miltenyi Biotec) by negative selection to reach more than 99% purity. Peripheral blood CD4⁺FOXP3⁺ Treg cells were purified from PBMCs by using the CD4⁺ T Cell Isolation Kit II (Miltenyi Biotec) and anti-CD25 mAb coupled to microbeads (Miltenyi Biotec).

Coculture experiments

Maturing tonsil pDCs and mDCs were cocultured with allogeneic naive CD4⁺ T cells at a 1:10 ratio for 6 days. As controls, naive CD4⁺ T cells supplemented with IL-2 (100 U/mL, Proleukin Proreco Pharma, Liestal, Switzerland) were cultured alone in complete RPMI. FOXP3 mRNA expression was quantified in primed T cells after 6 days of coculture without any further second stimulation. FOXP3, CD25, CD127, and CD4 expression in the primed T cells was determined by flow cytometry after 6 days of coculture without any second further stimulation. For all FOXP3 determinations, the 259D clone for the anti-FOXP3 mAb was used (BioLegend, Uithoorn, The Netherlands).

Treg-cell suppression assay

Unlabeled purified tonsil CD4⁺FOXP3⁺ Treg cells ("suppressor" cells) were mixed with carboxy-fluorescein succinimidyl ester (CFSE)-labeled autologous TMCs ("responder" cells) at different ratios and stimulated with plate-bound anti-CD3 antibody (1 μ g/mL) for 5 days. For control purposes, CFSE-labeled TMCs were cultured alone with or without plate-bound anti-CD3. Proliferation was determined by CFSE dilution by flow cytometry after staining with phycoerythrin and cyanin 5 (PC5)-conjugated anti-CD4. The same type of experiments were performed by using purified CD4⁺FOXP3⁺ Treg cells from peripheral blood as suppressor cells and CFSE-labeled autologous PBMCs as responder cells.

The induced CD4⁺FOXP3⁺ Treg cells by tonsil pDCs after 6 days of coculture (without second stimulation) were purified with the

CD4⁺CD25⁺CD127^{dim/-} Regulatory T Cell Isolation Kit II (Miltenyi Biotec). The induced CD25⁺ T cells by tonsil mDCs after 6 days of coculture (without second stimulation) were purified with anti-CD25 microbeads. FOXP3, CD25, CD127, and CD4 expression of the purified cells was determined by flow cytometry. Their suppressive capacity was also evaluated by CFSE dilution experiments as described above.

Statistical analysis

Data are expressed as means $\pm$ SEM. The Mann-Whitney *U* and Wilcoxon signed rank tests were used for statistical analysis. Correlation analysis was performed by Spearman's correlation test.

Further detailed methods on media and reagents, cell purification, cell cultures, flow cytometry, immune histology, RNA extraction, cDNA synthesis, quantitative real-time RT-PCR, and HLA-DR typing and staining with MHC class II peptide tetramers are described in this article's [Methods](#) section in the Online Repository at www.jacionline.org.

RESULTS

High numbers of functional CD4⁺FOXP3⁺ Treg cells in human palatine tonsils

The cell composition of TMCs showed a major difference in comparison with that of peripheral blood. The percentage of B cells in palatine TMCs was approximately 6 times higher than that of PBMCs, and the percentage of total T cells as well as the CD4⁺ and CD8⁺ T-cell compartments was significantly lower in TMCs (Fig 1, A). Although the frequency of T cells in tonsils was lower than in peripheral blood, the percentage of CD3⁺FOXP3⁺ and CD4⁺FOXP3⁺ Treg cells in TMCs was significantly high (Fig 1, B).

Tonsil CD3⁺CD4⁺FOXP3⁺ Treg cells expressed CD25, intracellular cytotoxic T lymphocyte-associated antigen 4, CD39, and CD62L as classical Treg-cell markers (Fig 1, C). Most of the tonsil CD4⁺FOXP3⁺ Treg cells showed a memorylike phenotype (CD45RO⁺), but a naive phenotype (CD45RA⁺) was also detectable in approximately 13% of the cells. A fraction of tonsil CD4⁺FOXP3⁺ Treg cells expressed inducible costimulator or program death 1, and very few had CD103 or CD127.

Only a small fraction of tonsil CD4⁺FOXP3⁺ Treg cells produced IFN- γ or IL-10 (Fig 1, D). Neither IL-4- nor IL-13-producing CD4⁺FOXP3⁺ Treg cells were identified in TMCs. Purified tonsil Treg cells showed strong suppressive capacity compared with Treg cells purified from peripheral blood. Purified tonsil CD4⁺FOXP3⁺ Treg cells and purified circulating CD4⁺FOXP3⁺ Treg cells failed to proliferate and suppressed the proliferation of anti-CD3-stimulated autologous TMCs or PBMCs, respectively, in a dose-dependent manner (Fig 1, E).

pDCs are the major DC subset in human palatine tonsils

Tonsil pDCs were identified as a homogeneous population of cells expressing the specific-surface markers CD123 and CD303 (0.57% $\pm$ 0.06%) (Fig 2, A). By using 2 specific-surface markers (CD1c and CD11c) and gating on CD19⁻ cells, at least 3 different populations of tonsil mDCs were identified in TMCs: (i) a subset expressing CD1c (0.21% $\pm$ 0.04%), (ii) a subset expressing CD11c (0.46% $\pm$ 0.05%), and (iii) a subset expressing both CD1c and CD11c (0.16% $\pm$ 0.05%). Remarkably, the percentage of tonsil pDCs was significantly higher and the percentage of

tonsil mDCs (the subset coexpressing CD1c and CD11c) was significantly lower than that of PBMCs. To determine the percentage of each subset in total TDCs, we obtained a fraction enriched in TDCs. pDCs represented the major DC subset in human palatine tonsils (65.5% $\pm$ 4.2%) (Fig 2, B).

FOXP3⁺ Treg cells colocalize with pDCs and proliferate in T-cell areas of human palatine and lingual tonsils

Although tonsillectomy takes place in palatine tonsils, the lingual tonsil that is in very close contact with all swallowed food remains lifelong intact. Both the lingual and palatine tonsils showed similar *in vivo* proliferating Treg cells colocalized with pDCs. FOXP3⁺ Treg cells and pDCs were identified in the T-cell areas but not in the B-cell areas of human lingual and palatine tonsils (Fig 3, A; see Fig E2, A, in this article's Online Repository at www.jacionline.org). FOXP3⁺ Treg cells and pDCs were in very close contact in the T-cell areas of human lingual and palatine tonsils in the proximity of crypt epithelia cells (Fig 3, B, and Fig E2, B). Approximately 40% of FOXP3⁺ Treg cells were in close contact with pDCs both in palatine and in lingual tonsils (see Fig E3, A, in this article's Online Repository at www.jacionline.org). Staining of palatine and lingual tonsil sections with FOXP3 and Ki-67 demonstrated the *in vivo* proliferation of FOXP3⁺ Treg cells in human tonsils (Fig 3, C, and Fig E2, C). Approximately 13% of FOXP3⁺ Treg cells proliferate both in palatine and in lingual tonsils (Fig E3, B).

Allergen-specific CD4⁺FOXP3⁺ Treg cells and allergen tolerance in tonsils

To demonstrate the existence of allergen-specific Treg cells in tonsils, we used MHC class II peptide tetramers for birch pollen major allergen Bet v 1 because of its very high cross-reactivity to many food and aeroallergens and relatively high expectance of specific T-cell frequency.³⁴⁻³⁶ Bet v 1-specific T cells were identified in freshly isolated TMCs with a frequency of approximately 0.38% of the total T helper cell population (Fig 4, A). Within these Bet v 1-specific T cells, the frequency of FOXP3⁺ Treg cells constituted approximately 30% of the population. The percentage of Bet v 1-specific CD4⁺ as well as Bet v 1-specific FOXP3⁺ Treg cells was significantly higher in freshly isolated TMCs than in PBMCs (Fig 4, A). Freshly isolated TMCs from an HLA-DRB1 negative donor (typed positive for *13/*14) was stained with all the tetramer molecules as negative controls (see Fig E4 in this article's Online Repository at www.jacionline.org). We further analyzed the frequency of tonsil Bet v 1-specific T cells on *in vitro* expanded populations. The percentage of tonsil Bet v 1-specific CD4⁺ T cells as well as their FOXP3 expression significantly increased after 14 days of *in vitro* expansion (Fig 4, B).

Tonsils showed a general T-cell tolerance with significantly low T-cell proliferation in response to common antigens and allergens, suggesting an *in vivo* control of any exaggerated enlargement due to their critical anatomical localization in the pharynx (Fig 5, A). This proliferation to any stimulus was controlled by Treg cells in general because proliferative responses of TMCs depleted of FOXP3⁺ Treg cells to allergens and other antigens were significantly higher than those observed in TMCs (Fig 5, A). Most of the FOXP3⁺ Treg cells were depleted from TMCs by using anti-CD25 microbeads without significantly altering the number

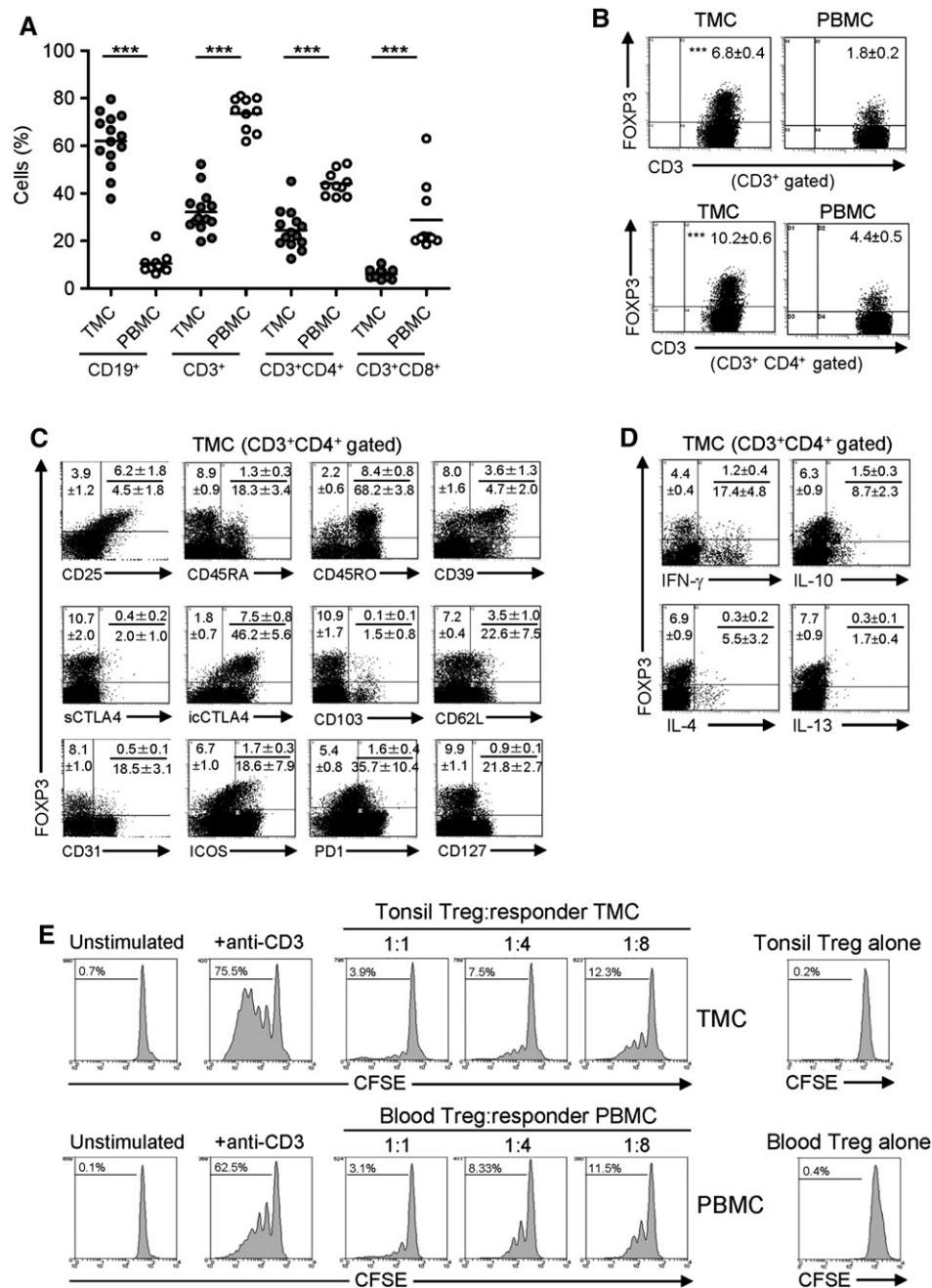


FIG 1. High numbers of functional CD4⁺FOXP3⁺ Treg cells in human palatine tonsils. **A**, Percentage of cells expressing the indicated surface markers in freshly isolated TMCs and PBMCs. **B**, FOXP3 T-cell expression in freshly isolated TMCs (n = 20) compared with PBMCs (n = 10). **C**, Characterization of CD3⁺CD4⁺FOXP3⁺ Treg cells in freshly isolated TMCs (n = 5). **D**, Dot plots of the indicated cytokines and FOXP3 in freshly isolated TMCs (n = 7) gated on CD3⁺CD4⁺ T cells. **E**, Equal suppressive capacity and no self-proliferation of tonsil and peripheral blood Treg cells. The histograms show the percentage of proliferating responder TMCs or PBMCs stimulated with plate-bound anti-CD3 for each assayed condition. Data are from 1 of at least 3 independent experiments with the same result. ***P < .0001.

of B cells, mDC subsets, or pDCs (see Fig E5, A and B, in this article's Online Repository at www.jacionline.org). This increased antigen-specific T-cell proliferation was restored to basal levels after adding back purified autologous tonsil FOXP3⁺ Treg cells (Fig 5, B), indicating the presence of functional FOXP3⁺ Treg cells in human palatine tonsils.

Tonsil pDCs induce CD4⁺FOXP3⁺ functional Treg cells

To study the phenotypic and functional properties of tonsil DCs, we purified tonsil pDCs and mDCs from TDCs (see Fig E6, A, in this article's Online Repository at www.jacionline.org). Purified tonsil pDCs stimulated for 24 hours with TLR7-L or

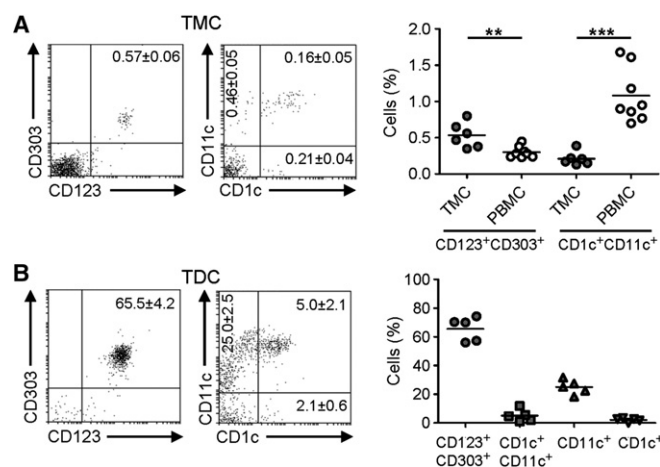


FIG 2. Human tonsils predominantly contain pDCs. **A**, CD123/CD303- and CD1c/CD11c-expressing DCs in freshly isolated TMCs. The graph displays the percentages of DC subsets in freshly isolated TMCs and PBMCs. $^{**}P < .001$, $^{***}P < .0001$. **B**, The pDC and mDC fractions enriched in TDCs were stained for CD123/CD303 and CD1c/CD11c. The graph displays the percentages of DC subsets in TDCs.

TLR9-L produced high amounts of IFN- α , IL-6, and TNF- α (Fig E6, B). Purified tonsil mDCs did not produce IFN- α after TLR2-L or TLR8-L stimulation and the levels of IL-6 and TNF- α were lower than those produced by tonsil pDCs. In contrast, tonsil mDCs produced large amounts of IL-10. The levels of IL-12 produced by tonsil mDCs activated via TLR8-L were much higher than after TLR2-L activation. Tonsil pDCs and mDCs expressed a premature state in human tonsils and acquired a mature phenotype after 24 hours of stimulation via TLRs with upregulation of HLA-DR, CD80, and CD83 (Fig E6, C).

FOXP3 mRNA levels were notably upregulated after 6 days of coculture without any second stimulation in T cells cocultured with IL-3, TLR7-L-, or TLR9-L-activated pDCs compared with TLR2-L- or TLR8-L-activated mDCs (see Fig E7, A, in this article's Online Repository at www.jacionline.org). After 6 days of coculture without any additional stimulation, most of the CD4⁺FOXP3⁺ Treg cells induced by IL-3-, TLR7-L-, or TLR9-L-activated tonsil pDCs expressed CD25 but not CD127 (Fig 6, A). The percentage of CD4⁺CD25⁺FOXP3⁺ T cells generated by TLR2-L- and TLR8-L-activated tonsil mDCs was considerably lower than that induced by tonsil pDCs (Fig 6, A). Purified tonsil DC subsets without any TLR or IL-3 stimulation died after 24 hours in cultures. Freshly isolated naive CD4⁺ T cells did not express CD25 or FOXP3 (Fig E7, B).

Purified CD4⁺FOXP3⁺ Treg cells induced by tonsil pDCs after 6 days of coculture without second stimulation (Fig 6, B) suppressed the proliferation of autologous T cells (Fig 6, C). There was no difference in the suppressive capacity of Treg cells generated by IL-3-, TLR7-L-, or TLR9-L-stimulated tonsil pDCs. After restimulation with anti-CD2/CD3/CD28 for 48 hours, only IL-10 but not IFN- γ , IL-4, or IL-5 mRNA levels were upregulated in CD4⁺ T cells primed by tonsil pDCs activated with IL-3, TLR7-L, or TLR9-L compared with naive CD4⁺ T cells cultured alone (Fig E7, C). Interestingly, most of the FOXP3⁺ Treg cells induced by tonsil pDCs did not produce IL-10 after restimulation with phorbol 12-myristate 13-acetate/ionomycin for 6 hours (Fig E7, D). In contrast, purified CD4⁺CD25⁺ T cells induced by tonsil mDCs after 6 days of coculture without second

stimulation (Fig 6, B) were not suppressive and displayed a hyperstimulatory capacity for T-cell proliferation (Fig 6, C).

The number of pDCs and FOXP3⁺ Treg cells significantly correlate in tonsils from nonatopic subjects

We quantified and compared the frequencies of FOXP3⁺ Treg cells and DC subsets in freshly isolated TMCs from 10 atopic and 15 nonatopic subjects. The percentage of FOXP3⁺ Treg cells was equally high in both groups (Fig 7, A). Although we did not find statistically significant differences between both groups when comparing different DC subsets, TMCs from atopic individuals contained lower frequencies of pDCs than did TMCs from nonatopic subjects. Conversely, the frequencies of all mDC subsets were higher in atopic than in nonatopic TMCs. Interestingly, when plotting the percentage of pDCs versus the percentage of FOXP3⁺ Treg cells in TMCs from atopic and nonatopic subjects, we found a highly significant correlation between the 2 cell types in nonatopic subjects but not in the group of atopic individuals (Fig 7, B). There was no correlation between the percentages of total mDCs and those of FOXP3⁺ Treg cells.

DISCUSSION

The present study shows that oral tolerance to common allergens through the generation of allergen-specific Treg cells is taking place in human tonsils and suggests a suitable lymphatic organ for direct immune interventions for the treatment of allergic diseases and other immune tolerance-related disorders. Because of their anatomical localization with unique structural and functional features, tonsils mount innate and adaptive immune responses that must discriminate between potentially dangerous pathogens and innocuous antigens.^{2,37} It has been shown that antacid treatments may induce allergic sensitization, suggesting that an allergen tolerance mechanism may have a role anatomically over the gastric acid and digestive proteases.³⁸ Accordingly, we hypothesized that tonsils might represent a suitable microenvironment for the generation of aeroallergen and food antigen-specific immune tolerance as they are the only lymphoid tissue that are exposed to the innocuous environmental antigens before being degraded by the digestive system. To evaluate this hypothesis, we investigated noninflamed palatine tonsils and lingual tonsils directly after tonsillectomy and focused to demonstrate the *in vivo* situation by direct analysis of allergen-specific T-cell responses. Our results showed that tonsils comprise a distinct cellular profile compared with peripheral blood. We found that the frequency of CD4⁺FOXP3⁺ Treg cells in tonsils was approximately 3 times higher than that in peripheral blood. Tonsil CD4⁺FOXP3⁺ Treg cells expressed classical Treg-cell surface markers,³⁹ and they did not express the alpha chain of the IL-7 receptor (CD127), which differentiates human regulatory cells from activated T cells.⁴⁰ They displayed a partially anergic status with low cytokine production, failed to proliferate after anti-CD3 stimulation, and exhibited equal T-cell suppressive capacity compared with Treg cells from peripheral blood, thus demonstrating that functional CD4⁺FOXP3⁺ Treg cells represent a main component in human palatine tonsils.

Next, we investigated whether a specific DC subset might be associated with the high frequency of Treg cells observed in palatine tonsils. We identified 4 DC populations in TMCs

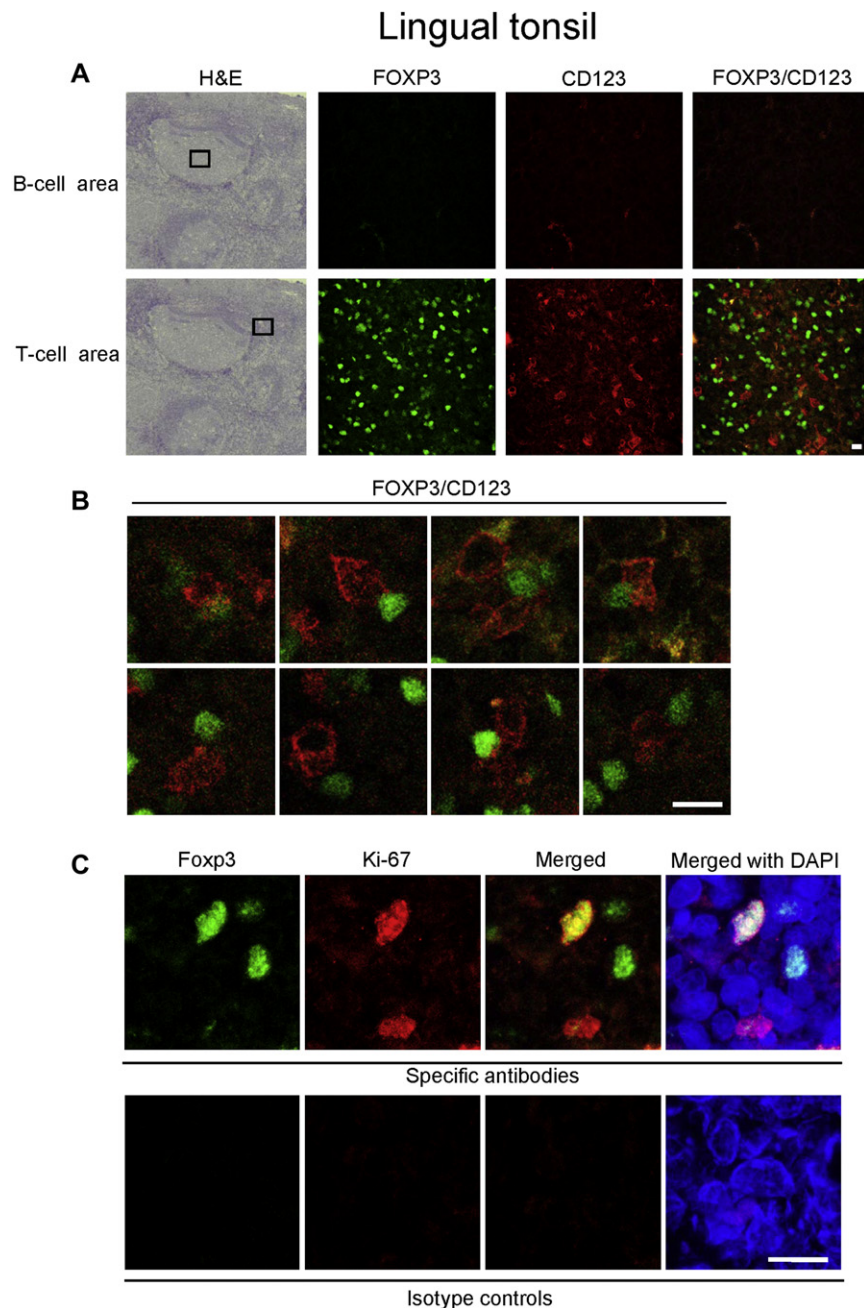


FIG 3. FOXP3⁺ Treg cells colocalize with pDCs in the T-cell areas of human lingual tonsils. **A**, The *black squares* inside H&E sections (*left panels*) indicate the B- and T-cell areas shown on the right panels. The sections were stained for FOXP3 (*green*) and CD123 (*red*) and analyzed by confocal microscopy. **B**, Close contact and direct interactions between FOXP3⁺ (*green*) Treg and CD123⁺ (*red*) pDCs. **C**, Sections were stained for FOXP3 (*green*), ki-67 (*red*), and DAPI (*blue*) or for the corresponding matching isotype controls. Data are from 1 of at least 3 tissue samples with similar results. *White bars*, 10 μ M. DAPI, 4'-6-Diamidino-2-phenylindole, dihydrochloride.

according to the differential expression of the DC specific-surface markers: CD123, CD303, CD1c, and CD11c. It is likely that more phenotypic subsets of DCs can be identified because of the usage of different battery of antibodies against specific-surface markers. We identified pDCs as the major DC subset in human palatine tonsils.^{30,32} To gain insight into the *in vivo* relevance of FOXP3⁺ Treg-cell generation and maintenance, we investigated the localization and *in vivo* proliferation of Treg

cells and pDCs in human palatine and lingual tonsils. Interestingly, approximately 40% of FOXP3⁺ Treg cells colocalize with pDCs in the extrafollicular T-cell areas of tonsils *in vivo*, thus suggesting that pDCs might contribute to keep homeostasis in human tonsils through the generation of Treg cells. Approximately 13% of FOXP3⁺ Treg cells were proliferating *in vivo* in palatine and lingual tonsils, thus providing evidence that tonsils are a suitable niche for the *in vivo* expansion of

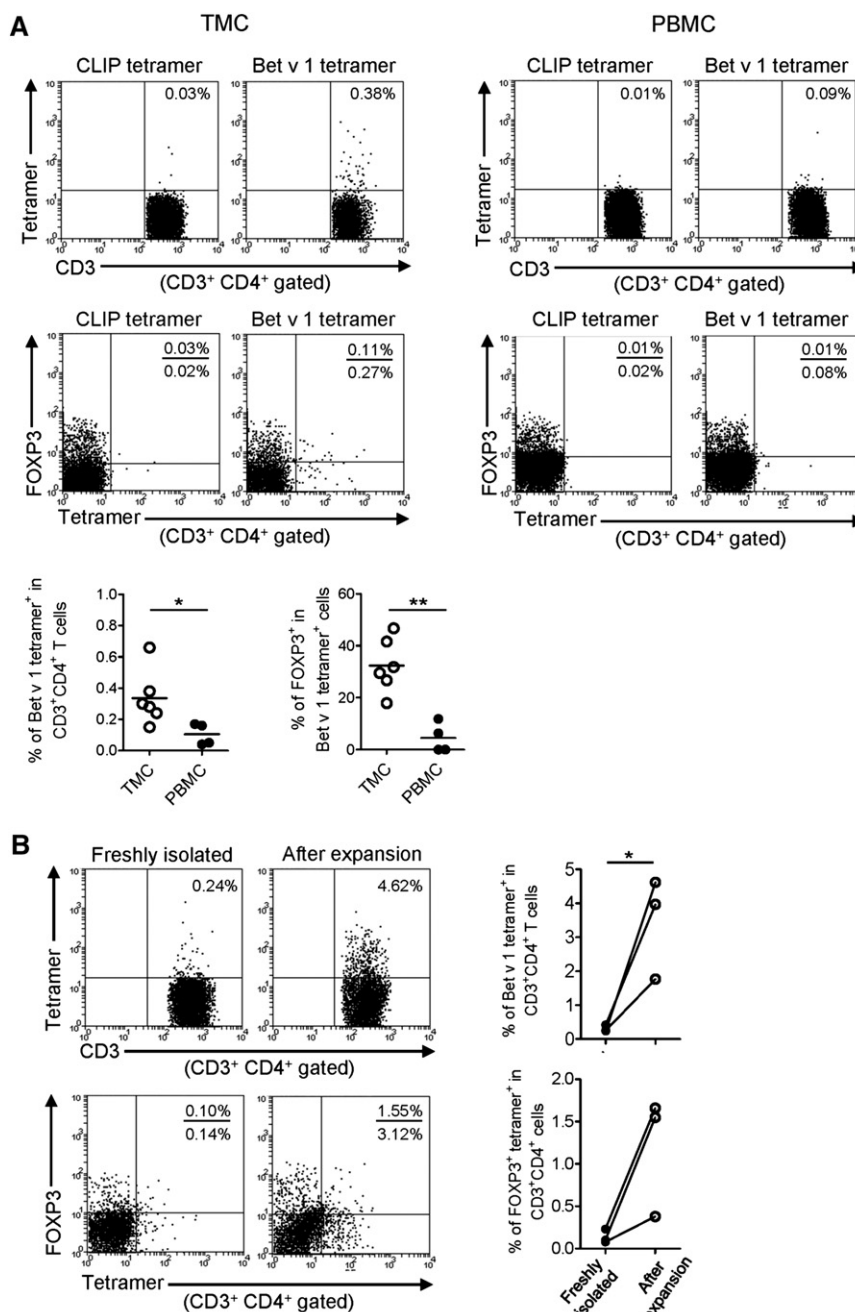


FIG 4. Detection of Bet v 1-specific FOXP3⁺ T cells in human palatine tonsils. **A**, Flow cytometric analysis of Bet v 1 tetramer⁺ CD4⁺ T cells and Bet v 1 tetramer⁺ FOXP3⁺ CD4⁺ T cells in freshly isolated TMCs and PBMCs. Class II-associated invariant chain peptide (CLIP) tetramers were used as negative control. The graphs display the percentage of Bet v 1 tetramer-positive T cells detected in CD3⁺CD4⁺ T cells and the percentage of FOXP3⁺ cells within the Bet v 1 tetramer⁺ CD4⁺ T cells. **P* < .05, ***P* < .005. **B**, Bet v 1 tetramer⁺ T cells and Bet v 1 tetramer⁺ FOXP3⁺ T cells within the CD3⁺CD4⁺ T cells in freshly isolated TMCs and after expansion of tonsil CD4⁺ T cells with Bet v 1. The graphs display the percentage of Bet v 1 tetramer⁺ T cells and the percentage of FOXP3⁺ tetramer⁺ cells in CD3⁺CD4⁺ T cells. **P* < .05.

FOXP3⁺ Treg cells. Considering that lingual tonsil is anatomically big, in full contact with all the swallowed food, and remains lifelong intact, our findings may shed light into the mechanisms operating as the first step of oral tolerance induction.

In the present study, we also demonstrated that purified tonsil pDCs and tonsil mDCs displayed a different cytokine signature

and acquired a maturing phenotype after TLR activation similar to peripheral blood pDCs and mDCs.^{27,41} Previous studies have reported that pDCs isolated from blood or thymus are able to generate Treg cells even in a maturing state.⁴²⁻⁴⁵ Our results demonstrated that tonsil pDCs stimulated with IL-3, TLR7-L, and TLR9-L induced CD4⁺FOXP3⁺ Treg cells with suppressive capacity *in vitro*. These data provide strong evidence to support

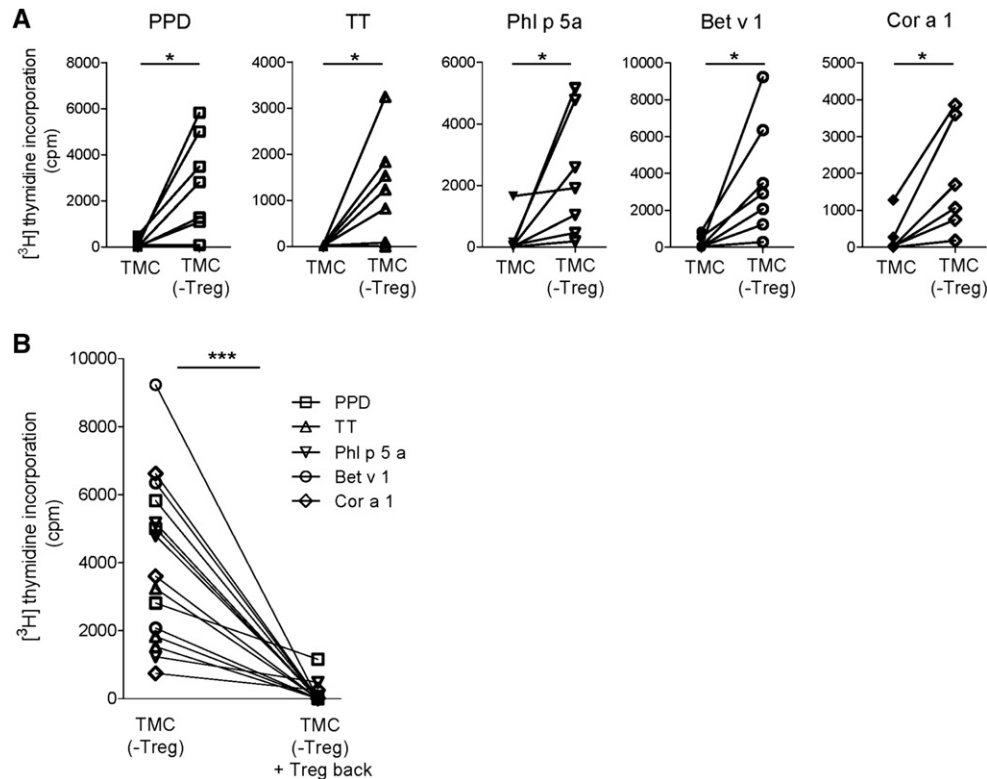


FIG 5. FOXP3⁺ Treg cells in immune tolerance to allergens in human tonsils. **A**, TMCs and TMCs depleted of FOXP3⁺ Treg cells (TMC [-Treg]) stimulated with the indicated antigens and allergens. **B**, TMC (-Treg) and TMC (-Treg) plus purified autologous tonsil FOXP3⁺ Treg cells stimulated with the indicated antigens and allergens. Proliferation was measured after 5 days of culture by [³H]-thymidine incorporation during the last 16 hours. The demonstrated counts per minute (cpm) values are those resulting after subtracting the cpm values of the corresponding unstimulated condition. **P* < .05, ****P* < .0005. PPD, Purified protein derivative; TT, tetanus toxoid.

that pDCs also play an important role in the generation of CD4⁺FOXP3⁺ Treg cells in human tonsils. Interestingly, we also demonstrated that the number of FOXP3⁺ Treg cells is directly proportional to the number of pDCs in human tonsils from nonatopic individuals, thus supporting the role of pDCs in the generation of Treg cells that may contribute to immune tolerance to allergens.

Induction of allergen-specific Treg cells with the capacity to suppress antigen both specifically and nonspecifically *in vitro* and *in vivo* plays a major role in a healthy nonallergic immune response to a high dose of allergen exposure, such as beekeepers exposed to bee venom,^{5,6} as well as in successful allergen-specific immunotherapy.¹⁶ We studied allergen-specific T-cell responses and the role of specific Treg cells in allergen tolerance in human palatine tonsils. TMCs did not show significant proliferative responses to common food and aeroallergens, thus reflecting an ongoing allergen-specific T-cell tolerance. Depletion of FOXP3⁺ Treg cells enabled allergen-induced proliferation of tonsil T cells. When purified autologous tonsil FOXP3⁺ Treg cells were added back to Treg-depleted responder cells, allergen-stimulated T-cell proliferation was inhibited again, indicating an active role of Treg cells in allergen-specific T-cell unresponsiveness. We detected Bet v 1-specific FOXP3⁺ Treg cells in freshly isolated TMCs by using MHC class II peptide tetramers, thus confirming that allergen-specific FOXP3⁺ Treg cells exist and play a role in controlling

allergen-specific T-cell responses in human tonsils. Several studies have reported that the frequency of circulating Bet v 1-specific CD4⁺ T cells is too low to be detected without *in vitro* expansion.⁴⁶ Here, we showed that tonsil Bet v 1-specific CD4⁺ T cells can be detected in freshly isolated TMCs and around 30% of these Bet v 1-specific cells are FOXP3⁺ Treg cells. The data clearly demonstrate an enrichment of allergen-specific T cells in tonsils in agreement with the demonstration of proliferating Treg cells inside the T-cell areas. In addition, we were able to expand *in vitro* the tonsil Bet v 1-specific CD4⁺ T cell as well as the Bet v 1-specific FOXP3⁺ Treg-cell populations. We cannot completely exclude that the observed increase in FOXP3 expression within the Bet v 1-specific T-cell population might also be partially due to the activation of Bet v 1-specific conventional T cells. However, the finding that FOXP3 expression is maintained after culturing the cells for 14 days in the presence of IL-2 without further antigen stimulation strongly suggests that the expanded cells are true Treg cells.

One of the major goals in allergy research is the design of novel immunotherapy protocols that lead to better therapeutic outcomes.⁴⁷ Direct injections of allergen extracts into lymph nodes represent a promising therapy with enhanced efficacy and safety as well as drastically reduced treatment time.⁴⁸ Our findings showing that tonsils are organs where Treg-cell generation occurs together with their privileged anatomical location suggest that

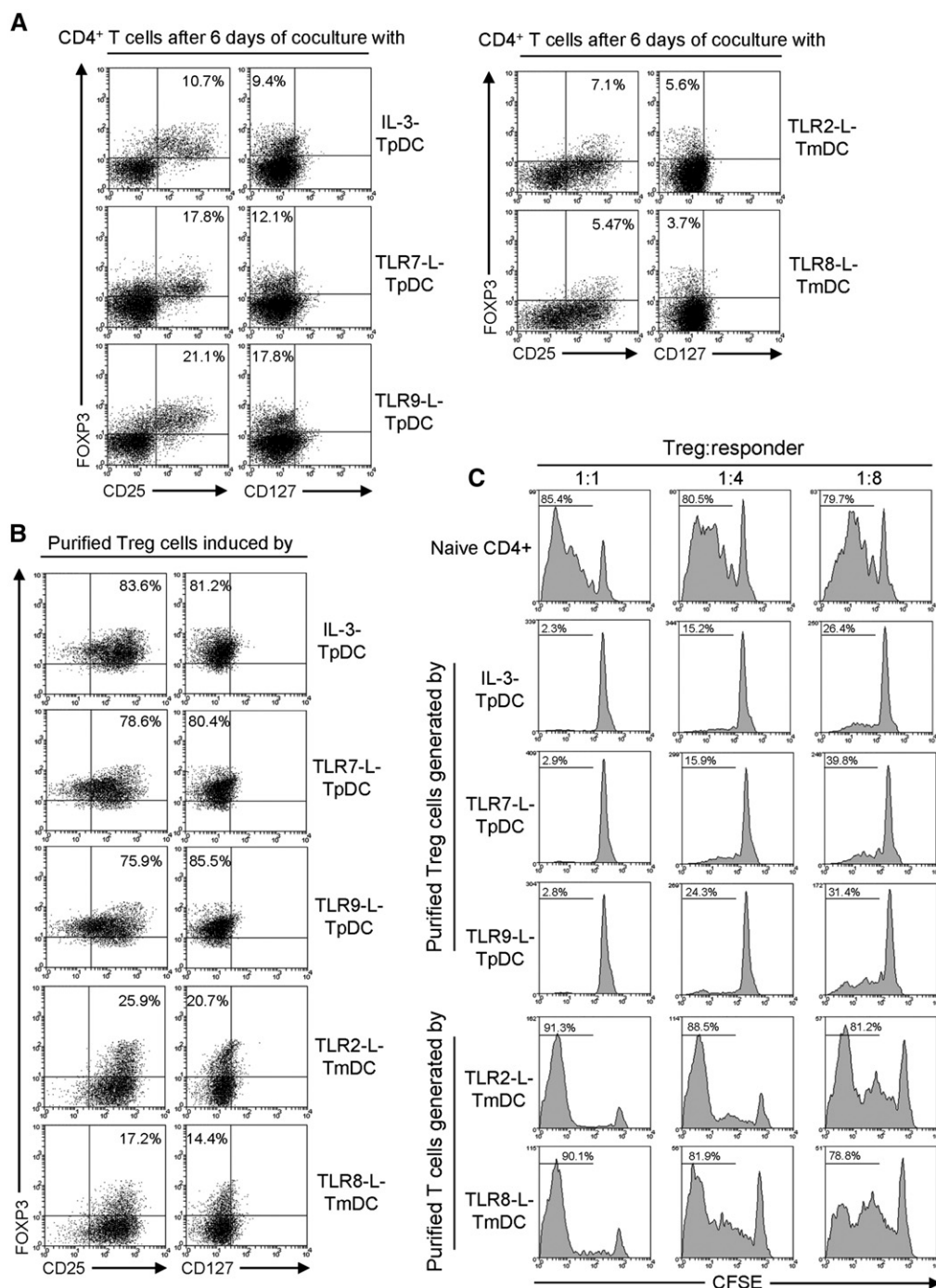


FIG 6. Tonsil pDCs drive naive CD4⁺ T cells to functional CD4⁺FOXP3⁺ T cells. **A**, Flow cytometric analysis of FOXP3/CD25 and FOXP3/CD127 expression in CD4⁺ T cells after 6 days of coculturing allogeneic naive CD4⁺ T cells with TpDCs or TmDCs activated as indicated. **B**, FOXP3/CD25 and FOXP3/CD127 expression of purified Treg cells induced by TpDCs and purified CD4⁺CD25⁺ T cells induced by TmDCs. One representative experiment out of 3 with similar results is displayed. **C**, Proliferation of CFSE-labeled PBMCs gated on CD4⁺ cells after 5 days of coculture with autologous purified CD4⁺FOXP3⁺ Treg cells generated by allogeneic TpDCs or TmDCs. The histograms show the percentage of proliferating responder cells stimulated with plate-bound anti-CD3. Naive CD4⁺ cells cultured without DC were used for control purposes. One representative experiment out of 3 with similar results is displayed. *TmDC*, Tonsil myeloid dendritic cells; *TpDC*, tonsil plasmacytoid dendritic cells.

human palatine tonsils are excellent candidates to be used as target tissues for the induction of peripheral tolerance. Recent studies have demonstrated that in rhesus monkeys tonsillar

applications of attenuated Simian immunodeficiency virus induce protection in the animals against the rectal challenge of wild-type Simian immunodeficiency virus.^{49,50} Therefore, the rational

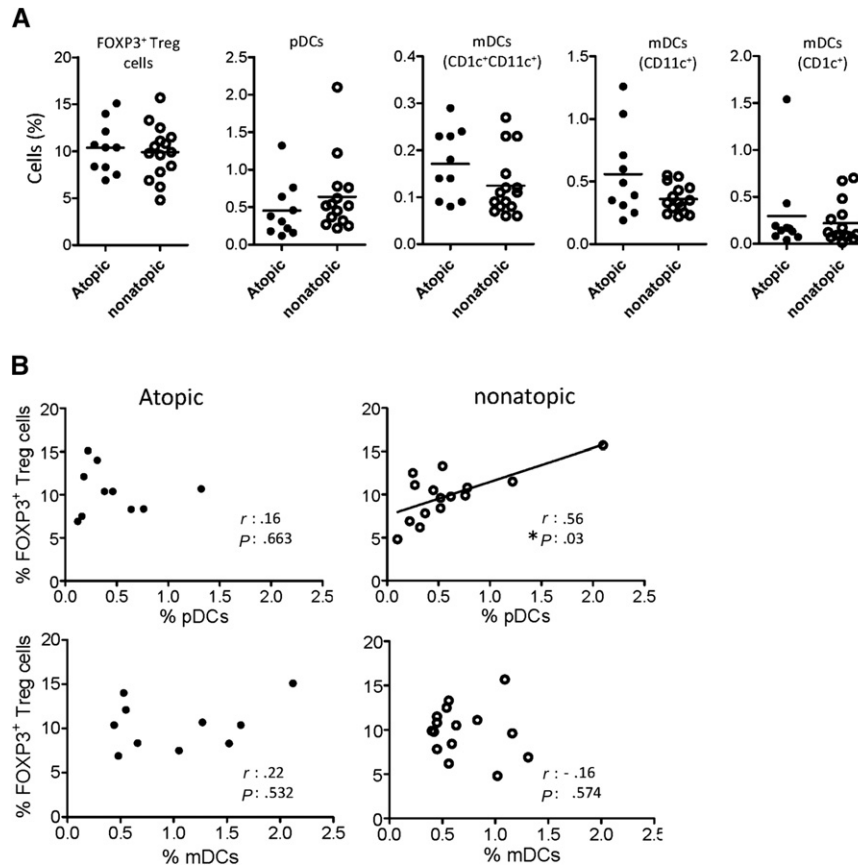


FIG 7. The frequencies of FOXP3⁺ Treg cell and DC subsets in tonsils from atopic and nonatopic individuals. **A**, Percentages of FOXP3⁺ Treg cells and DC subsets in TMCs from atopic and nonatopic individuals. **B**, Correlation analysis of the percentages of FOXP3⁺ Treg cells and pDCs or mDCs in TMCs from atopic and nonatopic individuals (* $P < .05$).

design of novel vaccines directly administered into human tonsils might open new avenues in the future not only for the treatment of allergic diseases but also for other immune tolerance-related disorders.

We thank Helmut Fiebig (Allergopharma Joachim Ganzer KG) for recombinant allergens.

Clinical implications: The demonstration of T-cell tolerance to allergens taking place in human tonsils suggests tonsils as target organs to develop novel ways of specific immunotherapies for the treatment of allergic and other immune tolerance-related diseases.

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METHODS

Media and reagents

RPMI 1640 supplemented with 5% heat-inactivated FCS (Amimed, Allschwil, Switzerland), antibiotics (penicillin, streptomycin, kanamycin from Life Technologies, Basel, Switzerland; amphotericin B from Bristol-Myers Squibb, Baar, Switzerland; and ciprofloxacin from Fluka, Buchs, Switzerland), MEM vitamin, L-glutamine, nonessential amino acids, and sodium pyruvate (Life Technologies) was used during the isolation of TMCs. The same medium was used for cell cultures without amphotericin B and ciprofloxacin and supplemented with 10% FCS. For the stimulation of tonsil plasmacytoid dendritic cells (TpDCs) and tonsil myeloid dendritic cells (TmDCs), we employed 10 ng/mL of IL-3 (PeproTech EC, London, United Kingdom), 2 μ M of TLR7-L Gardiquimoid (InvivoGen, Nunningen, Switzerland), 2 μ M of TLR9-L type B CpG2006 (Mycosynth, Balgach, Switzerland), 0.6 μ M of TLR2-L Pam3 Cys-Ser-(Lys)4 (Calbiochem, Zug, Switzerland), or 1 μ M of TLR8-L CL075 (3M002) (InvivoGen). For antigen-specific proliferation assays, the following allergens were used: 1 μ g/mL of rBet v 1 of birch pollen (*Betula verrucosa*), 1 μ g/mL of rPhl p 5a of timothy grass pollen (*Phleum pratense*), and 1 μ g/mL of rCor a 1 of hazelnut (*Corylus avellana*), all of them from Allergopharma Joachim Ganzer KG, Reinbek, Germany. Purified protein derivative and tetanus toxoid were used at 0.2 μ g/mL and 0.8 U/mL, respectively (both from Serum Institute, Copenhagen, Denmark). None of the reagents contained detectable amounts of LPS by Limulus assay (Cambrex Bio Science, Rockland, Me), and the purity of the recombinant allergens was more than 99%.

Purification of tonsil DC subsets

TmDCs were obtained from TDCs by positive selection by using the CD11c (BDCA-1)⁺ dendritic cell isolation kit (Miltenyi Biotec) supplemented in the first incubation step with CD11c-biotin antibodies. The negative fraction was used to purify TpDCs by positive selection by using the CD304 (BDCA-4/Neuropilin-1) microbead kit (Miltenyi Biotec).

Cell cultures

Purified TpDCs and TmDCs were seeded at a density of 2×10^5 cells/200 μ L of complete RPMI in flat-bottom 96-well plates with the corresponding activation stimulus for 24 hours at the concentration indicated above for each condition. After 24-hour stimulation, the activation surface markers HLA-DR, CD80, and CD83 were measured by flow cytometry and the supernatants were collected for cytokine determinations. Concentrations of IL-6, TNF- α , IL-10, and IL-12 in the cell-free supernatants were determined by using the cytometric bead array (Bio-Rad, Basel, Switzerland), according to the manufacturer's protocol. IFN- α levels were determined by using the human Verikine IFN- α ELISA kit (PBL Interferon Source, Piscataway, NJ) following the manufacturer's instructions.

For proliferation assays, TMCs (2×10^5 cells in 200 μ L) were cultured in medium alone or in the presence of antigens and allergens at the final concentrations described above for 5 days. In some allergen-specific proliferation experiments, FOXP3⁺ Treg cells were previously depleted from TMCs by using specific CD25 microbeads and autoMACS magnetic separation system (Miltenyi Biotec) following the manufacturer's indications. Purified autologous CD4⁺FOXP3⁺ Treg cells (2×10^4 cells) were added back to TMCs depleted of FOXP3⁺ Treg (2×10^5 cells) in the presence of allergens and antigens for stimulation. Proliferation was measured by adding [³H]-thymidine during the last 16 hours of culture.

RNA extraction, cDNA synthesis, and quantitative real-time RT-PCR

Total RNA was isolated by using the RNeasy mini kit (Qiagen, Hombrechtikon, Switzerland). Reverse transcription was performed with the Revert Aid M-MuLV Reverse Transcriptase (Fermentas, Nunningen, Switzerland) by using random hexamer primers. Gene expression was analyzed by quantitative real-time PCR by using iTaq SYBR Green Supermix with ROX (Bio-Rad) on a 7900HT Fast Real-Time PCR instrument (Applied

Biosystems, Foster City, Calif). The sequences of the employed primer pairs were as follows: Elongation factor 1 α (fwd, CTGAACCATCCAAT; rev, GCCGTGTGGCAATCCAAT) and FOXP3 (fwd, GAAACAGCACATTCC CAGAGTTC; rev, ATGGCCCAGCGGATGAG). PCR conditions were 10 minutes at 95°C followed by 40 cycles of 15 seconds at 90°C and 1 minute at 60°C. Relative quantification was performed by using the comparative $\Delta\Delta$ CT method.¹¹

Flow cytometry

The following mAbs were used for flow cytometry: fluorescein isothiocyanate-conjugated anti-CD123 (Miltenyi Biotec); anti-CD19 and anti-HLA-DR (Beckman Coulter); phycoerythrin (PE)-conjugated anti-CD45RA, anti-CD45-RO, anti-CD62L, and anti-CD127 (Beckman Coulter, Fullerton, Calif); anti-CD103 and anti-CD25 (Dako, Glostrup, Denmark); anti-CD39 (Ancell, Bayport, Minn); anti-inducible costimulator (eBioscience, Vienna, Austria); anti-CD31, anti-cytotoxic T lymphocyte-associated antigen 4, anti-PD1, anti-IFN- γ , anti-IL10, anti-IL-13, and anti-IL-4 (BD Pharmingen, San Diego, Calif); anti-CD303 and anti-CD1c (Miltenyi Biotec); energy-coupled dye-conjugated anti-CD4 and anti-CD8 (Beckman Coulter); PC5-conjugated anti-CD19, anti-CD3, and anti-CD25 (Beckman Coulter); and anti-CD11c, anti-CD80, and anti-CD83 (BD Pharmingen).

For analysis of FOXP3 expression in T cells primed with TpDCs or TmDCs at the single-cell level, cells were first subjected to surface staining with PC5-conjugated anti-CD3 and energy-coupled dye-conjugated anti-CD4 (Beckman Coulter). After fixation and permeabilization, the cells were stained with Alexa Fluor 488-conjugated mouse IgG1 anti-FOXP3 (Biolegend, Uithoorn, The Netherlands) following the manufacturer's recommendations. An Alexa Fluor 488-conjugated mouse IgG1 (Biolegend) was used as isotype control. The same protocol described above was carried out for the phenotypic characterization of the FOXP3⁺CD4⁺ Treg cells in freshly isolated TMCs. The corresponding PE-conjugated antibody in each case was added during the first surface staining step. For cytokine costaining of CD4⁺FOXP3⁺ Treg cells in fresh TMCs, the corresponding PE-conjugated anticytokine antibodies were added simultaneously with the Alexa Fluor 488-conjugated anti-FOXP3. The corresponding isotype controls were included in all stainings. Flow cytometric analysis was performed by an EPICS XL-MCL (Beckman Coulter) and further by Weasel v2.5 software and the CXP software (Beckman Coulter).

Immunohistochemistry

Palatine tonsil tissues were embedded in Tissue-Tek (Sakura Finetek, Alphen aan den Rijn, The Netherlands), frozen in liquid nitrogen-cooled 2-methylbutane (Fluka), and stored at -80°C until cryosections were cut by using an HM 500 OM microtome (Mikrotom, Nuremberg, Germany). Paraformaldehyde-fixed cryosections (7 μ) were sequentially stained with the following antibodies. First, the slides were incubated with antihuman CD123 mouse IgG1 mAb or the corresponding isotype control (eBioscience). After washing, CD123-binding antibodies were detected by using Alexa Fluor 532-conjugated goat antimouse IgG1 (Invitrogen, Basel, Switzerland). The sections were washed and a blocking step with mouse IgG1 mAb was included. Finally, the sections were stained with Alexa Fluor 488-conjugated mouse IgG1 mAb to FOXP3 or the proper isotype control (Biolegend). Tissue sections were mounted with ProLong Gold antifade reagent with 4'-6-diamidino-2-phenylindole, dihydrochloride (Invitrogen) for nuclei demonstration. Formalin-fixed, paraffin-embedded lingual tonsil sections (7 μ m) underwent deparaffinization and rehydration followed by antigen unmasking in Dako Target Retrieval Solution (Dako). Then, the sections were sequentially stained by using the same procedure described above. Images were acquired and analyzed by using the confocal microscope Leica DMI 4000B and the Leica TSC SPE system (Leica Microsystems GmbH, Glatbrugg, Switzerland). To identify proliferating FOXP3⁺ Treg cell in tonsils, we used antihuman ki-67 mouse IgG1 mAb (Santa Cruz, Heidelberg, Germany) and Alexa Fluor 532-conjugated goat antimouse IgG1 in combination with FOXP3 staining as above following the same sequential staining procedure described above. The general histology of palatine tonsils was demonstrated by staining of the sections with H&E.

HLA-DR typing and staining with MHC class II peptide tetramers

To identify donors expressing HLA-DRB1*1101, HLA-DRB1*1501, or DRB1*0701 alleles, HLA-DR typing was performed. First, genomic DNA was isolated from TMCs or PBMCs by using the QIAamp DNA Micro Kit (Qiagen). HLA-DRB1 allele specificity was determined by PCR sequence-specific primers in 2 consecutive steps. Low-resolution (2-digit) typing was first determined by using the Histo Type DR mini kit (BAG Healthcare, Lich, Germany). Those individuals showing HLA-DRB1*11, HLA-DRB1*15, or HLA-DRB1*07 were further analyzed for high-resolution (4-digit) typing by using DRB1*11, DRB1*15, and DRB1*07 SSP UniTray kits from Invitrogen. In this study, specific HLA-DRB1*1101, HLA-DRB1*1501, and HLA-DRB1*0701 Bet v 1 peptide tetramers and the corresponding CLIP peptide tetramers were employed to identify and quantify Bet v 1-specific FOXP3⁺ Treg cells in human palatine tonsils. Bet v 1 peptide sequences coupled to the tetramer molecules were Bet v 1₁₁₂₋₁₂₄ (GSILKISNKYHTK) for HLA-DRB1*1101 and Bet v 1₁₄₂₋₁₅₄ (ETLLRAVESYLLA) for HLA-DRB1*0701 and HLA-DRB1*15. In all cases, the corresponding tetramer molecules coupled to the CLIP (KMRMATPLLMQALPM) were used as negative controls. All the tetramers employed were labeled with PE. Freshly isolated TMCs

(2×10^6 cells) from 6 donors with specific alleles for DRB1*1101 (1 donor) or DRB1*0701 (5 donors) and freshly isolated PBMCs from 4 donors with specific alleles for DRB1*1501 (1 donor) or DRB1*0701 (3 donors) were stained with Bet v 1 tetramer molecules or the corresponding CLIP tetramers for 2 hours at 37°C in cRPMI. Next, the cells were stained with PC5-conjugated antihuman CD3, energy-coupled dye-conjugated antihuman CD4, and Alexa Fluor 488-conjugated antihuman FOXP3 as previously described. Cells were analyzed by flow cytometry. For the *in vitro* expansion of Bet v 1-specific T cells, CD4⁺ T cells were obtained from freshly isolated TMCs by using the CD4⁺ T Cell Isolation Kit II (Miltenyi Biotec). The obtained negative fraction (tonsil CD4⁺ T cells) was stimulated in 24-well plates with irradiated autologous TMCs (ratio 1:1) in cRPMI at 37°C in the presence of Bet v 1 (10 µg/mL). After 7 days, cell cultures were supplemented with 100 U/mL of IL-2 (ProleukinProreoPharma, Liestal, Switzerland) every second day. On day 14, cells were stained with Bet v1 tetramer molecules or the corresponding CLIP and FOXP3 as described above.

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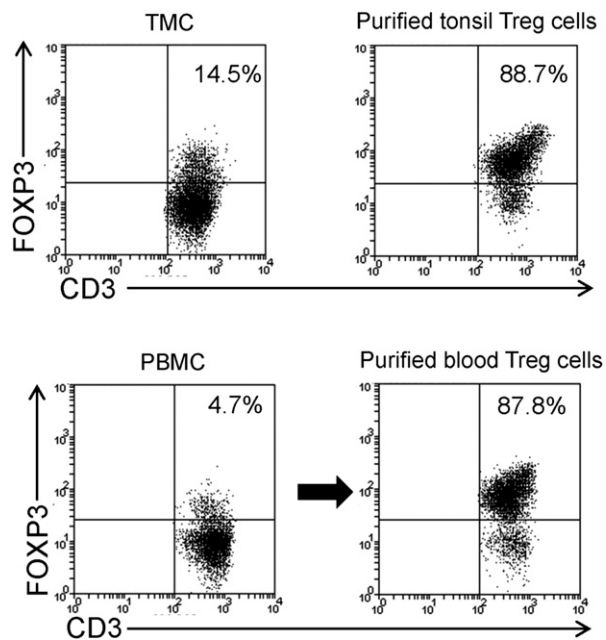


FIG E1. Purity of FOXP3⁺ Treg cells purified from tonsils and peripheral blood. Representative dot plots of CD3 and FOXP3 gating on CD3⁺CD4⁺ T cells in freshly isolated TMCs and the corresponding purified tonsil FOXP3⁺ Treg cells or freshly isolated PBMCs and the corresponding purified blood FOXP3⁺ Treg cells are shown. The percentage of FOXP3⁺ cells is displayed inside the quadrant.

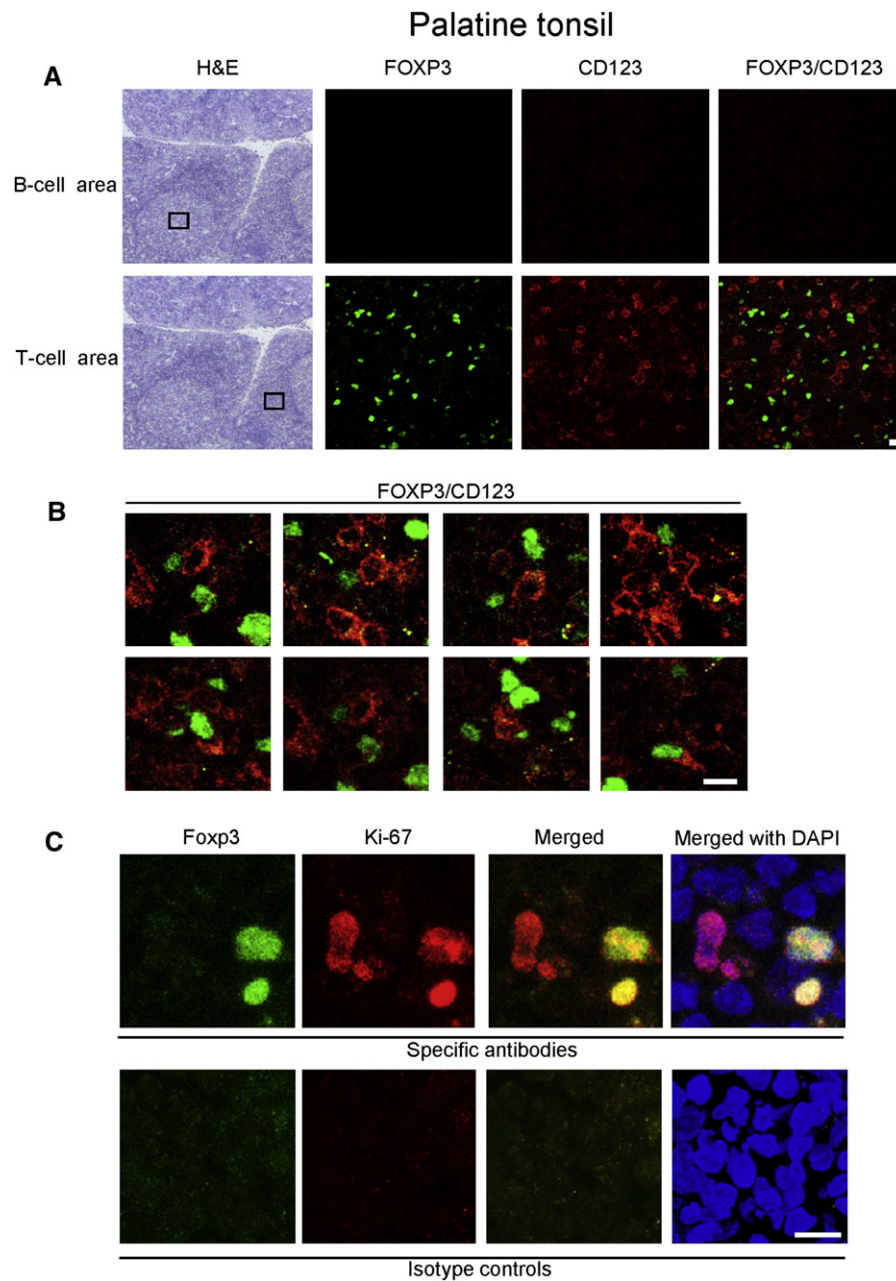


FIG E2. FOXP3⁺ Treg cells colocalize with pDCs in the T-cell areas of human palatine tonsils. **A**, The *black squares* inside H&E sections (*left panels*) indicate the B- and T-cell areas shown on the right panels. The sections were stained for FOXP3 (*green*) and CD123 (*red*) and analyzed by confocal microscopy. **B**, Close contact and direct interactions between FOXP3⁺ (*green*) Treg and CD123⁺ (*red*) pDCs. **C**, Sections were stained for FOXP3 (*green*), ki-67 (*red*), and DAPI (*blue*) or for the corresponding matching isotype controls. Data are from 1 of at least 3 tissue samples with similar results. *White bars*, 10 μ M. *DAPI*, 4'-6-Diamidino-2-phenylindole, dihydrochloride.

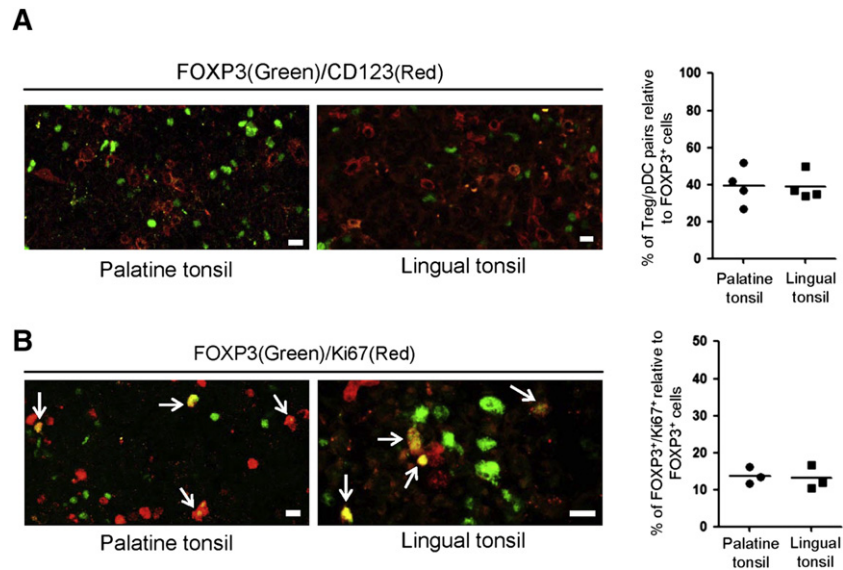


FIG E3. FOXP3⁺ Treg cells colocalize with pDCs and proliferate in T-cell areas of human tonsils. **A**, Human palatine and lingual tonsil sections stained for FOXP3 (green) and CD123 (red) and analyzed by confocal microscopy. The graph displays the percentage of FOXP3⁺ Treg/pDC (CD123⁺) pairs relative to the total FOXP3⁺ Treg cell population. **B**, Human palatine and lingual tonsil sections stained for FOXP3 (green) and ki-67 (red). Coexpression of both markers (yellow cells) is indicated by white arrows. The graph displays the percentage of FOXP3⁺/Ki67⁺ Treg cells relative to the total Treg-cell population. Data are from 1 of at least 3 to 4 tissue samples with similar results. White bars, 10 μ M.

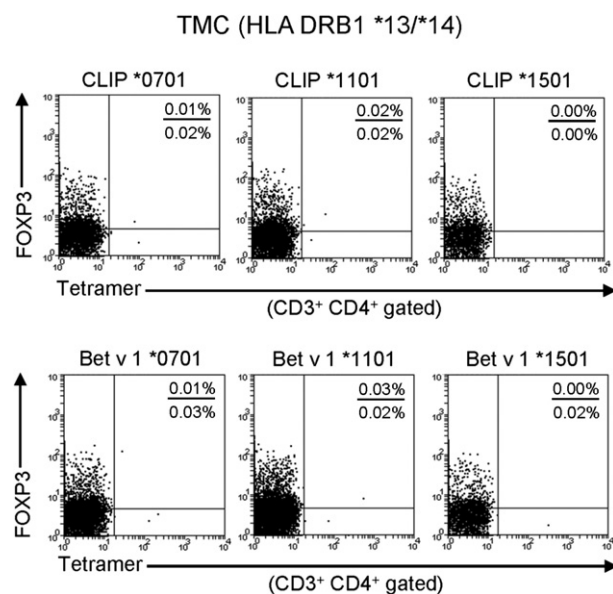


FIG E4. Control tetramer and FOXP3 staining in freshly isolated TMCs from a nonmatching donor for allele specificity. Representative flow cytometry dot plots of the staining with HLA-DRB1*0701, *1101, and *1501 CLIP peptide or Bet v 1 peptide tetramers in combination with FOXP3 in freshly isolated TMCs from a HLA-DRB1*13/*14 typed donor.

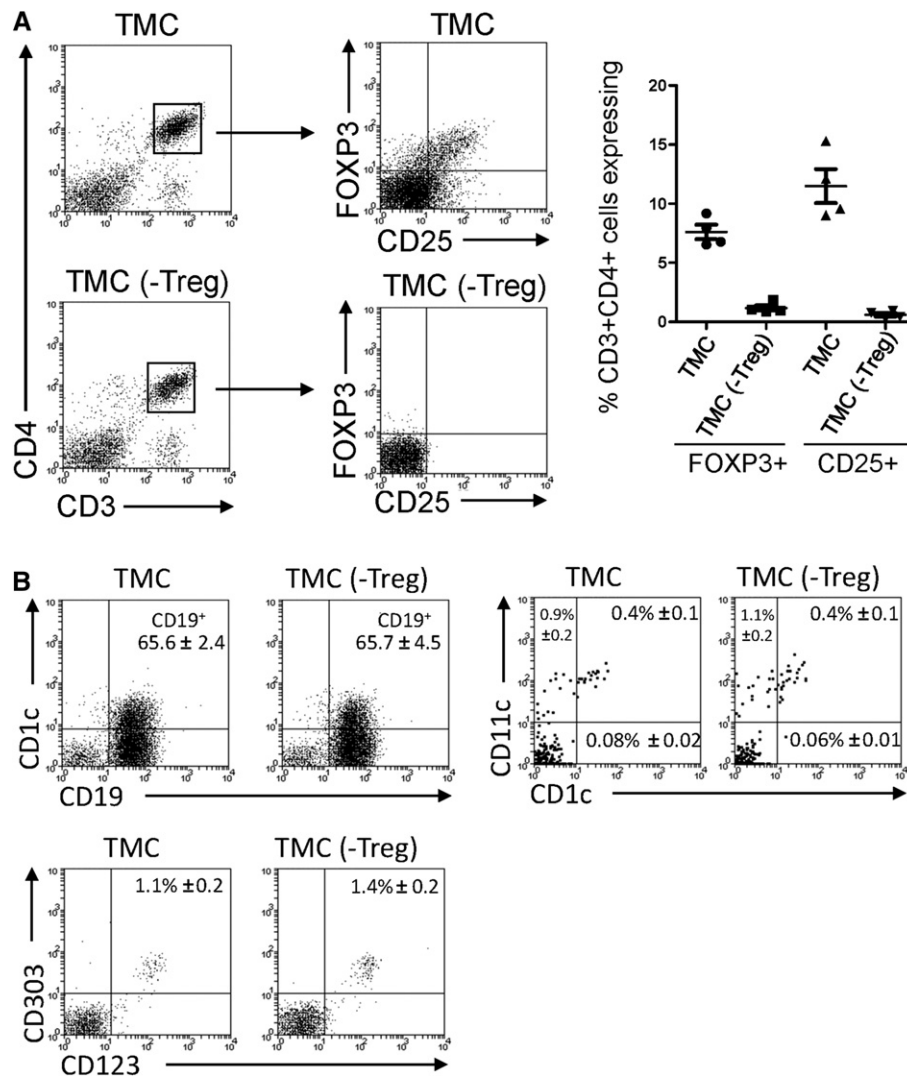


FIG E5. Depletion of FOXP3⁺ Treg cells from TMCs. **A**, Representative dot plots of TMCs and TMCs depleted of FOXP3⁺ Treg cells. On the left side, representative dot plots of CD3 and CD4 expression in TMCs and TMCs depleted of Treg cells are shown. The *black squares* indicate the CD3⁺CD4⁺ T-cell fraction gated for the subsequent analysis of FOXP3 and CD25 expression (representative dot plots are on the right side). The graph displays the percentage of FOXP3⁺ and CD25⁺ within the CD3⁺CD4⁺ T-cell population in TMCs and TMCs depleted of Treg cells (n = 4). **B**, Representative dot plots of TMCs and TMCs depleted of FOXP3⁺ Treg cells for B cells (CD19 vs CD11c), mDC subsets (CD11c vs CD11c), and pDCs (CD123 vs CD303). Percentages of B cells, mDC subsets, and pDCs are displayed inside the plots (n = 4).

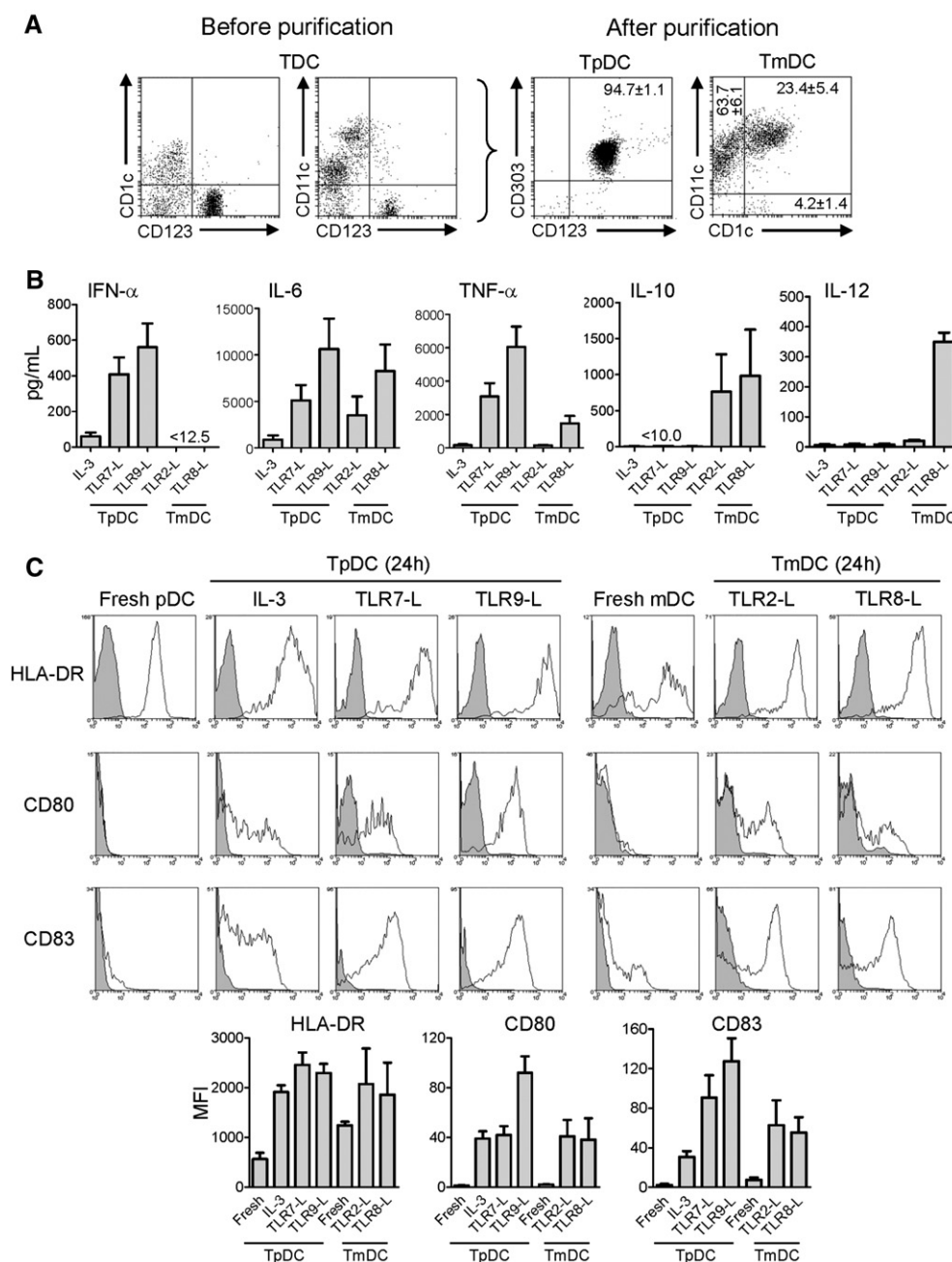


FIG E6. Purification and characterization of tonsil pDCs and mDCs. **A**, Different DC subsets can be visualized in the TDC fraction. On the *right side*, representative dot plots of CD123/CD303 of the purified TpDC and CD1c/CD11c of the obtained TmDC fraction are shown. The mean frequency ($\pm$ SEM) of cells expressing the indicated markers is also displayed. **B**, Purified TpDCs and TmDCs were stimulated for 24 hours with the indicated stimulus. Cytokines levels in cell-free supernatants were quantified by cytometric bead array (IL-6, TNF- α , IL-10, and IL-12) or by ELISA (IFN- α). Data represent the mean ($\pm$ SEM) of 3 to 6 independent experiments. **C**, The surface expression of HLA-DR, CD80, and CD83 on freshly purified TpDCs or TmDCs and TpDCs or TmDCs activated with the indicated stimulus for 24 hours was determined by flow cytometry. Histograms for a representative experiment are shown. Open histograms represent staining of the indicated marker, and closed histograms represent the matching isotype control. The graphs below display the mean fluorescence intensity using the entire cell population for each surface marker at different conditions. The data represent the mean $\pm$ SEM of 3 independent experiments.

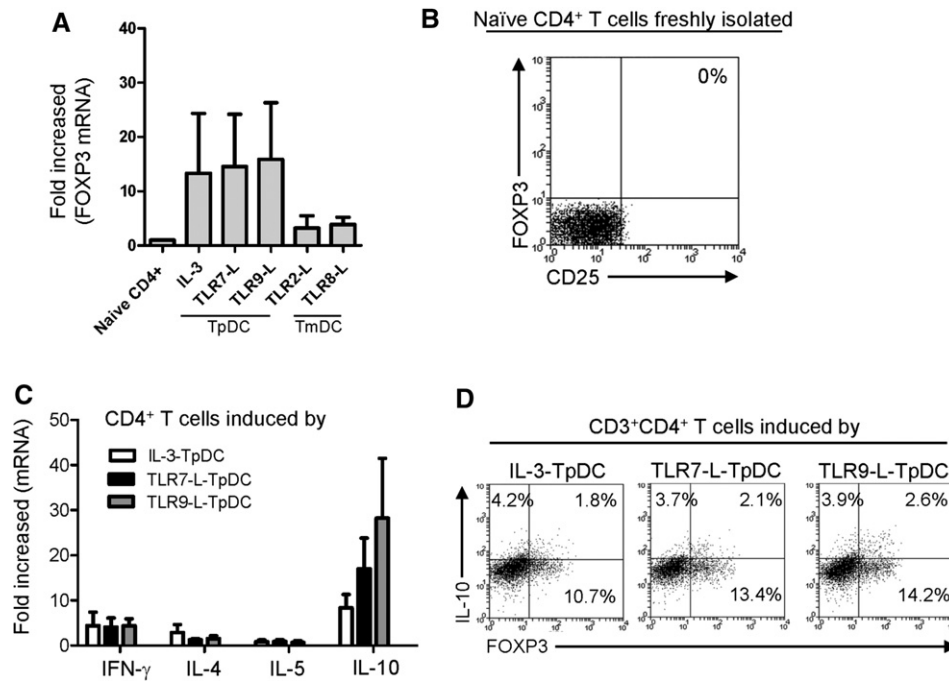


FIG E7. FOXP3⁺ Treg cells induced by activated TpDCs do not produce cytokines. **A**, FOXP3 mRNA expression determined by quantitative real-time RT-PCR after 6 days of coculturing allogeneic naive CD4⁺ T cells with TpDCs or TmDCs activated as indicated without any second further stimulation, $n = 3$. **B**, Representative flow cytometry dot plots of FOXP3/CD25 expression in the freshly isolated allogeneic naive CD4⁺ T cells used for coculture experiments with TpDCs and TmDCs. **C**, After 6 days of coculturing allogeneic naive CD4⁺ T cells with TpDCs (activated as indicated), primed T cells were washed and polyclonally stimulated with a mixture of soluble anti-CD2/CD3/CD28 mAbs for 48 hours. IFN- γ , IL-4, IL-5, and IL-10 mRNA expression was determined by using quantitative real-time RT-PCR. **D**, After 6 days of coculturing allogeneic naive CD4⁺ T cells with TpDCs activated as indicated, primed T cells were washed and stimulated with phorbol 12-myristate 13-acetate/ionomycin for 6 hours. FOXP3 and IL-10 expression determined by flow cytometry in CD3⁺CD4⁺ T cells. One representative example out of 3 with similar results is displayed.