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Nasal tolerance induces antigen-specific $CD4^+CD25^-$ regulatory T cells that can transfer their regulatory capacity to naive $CD4^+$ T cells

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Abstract

The mucosal immune system is uniquely adapted to elicit immune responses against pathogens but also to induce tolerogenic responses to harmless antigens. In mice, nasal application of ovalbumin (OVA) leads to suppression of both T_h1 and T_h2 responses. This tolerance can be transferred to naive mice by $CD4^+$ T_r cells from the spleen. Using the allotypic Ly5 system, we were able to demonstrate *in vivo* that T_r cells not only suppress naive $CD4^+$ T cells, but also induce them to differentiate into T_r cells. The effector function of these mucosal T_r cells is not restricted by cytokine polarization, since T_r cells from T_h1-tolerant mice can suppress a T_h2 response and *vice versa*. Transfer of splenic $CD4^+CD25^+$ and $CD4^+CD25^-$ T cell subsets from OVA-tolerized mice revealed that both subsets were equally able to suppress a delayed-type hypersensitivity response in acceptor mice. In contrast to the $CD25^-$ T cell subset, the $CD25^+$ cells were not specific for the antigen used for tolerization. Together, these findings demonstrate a role for $CD4^+CD25^-$ T_r cells in mucosal tolerance, which suppresses $CD4^+$ T cells in an antigen-specific fashion, irrespective of initial T_h1/T_h2 skewing of the immune response. This offers a major advantage in the manipulation of mucosal tolerance for the treatment of highly cytokine-polarized disorders such as asthma and autoimmune diseases.

Introduction

A characteristic feature of the mucosal immune system is its ability to tailor the immune response to the type of antigen that is encountered. As a result, pathogens generally elicit an active mucosal response, whereas innocuous antigens are generally tolerated. Mucosal tolerance, characterized by specific immunologic unresponsiveness to subsequent antigenic challenge, may be achieved by either the induction of anergy, deletion of responsive cells or active cellular regulation via T_r cells (1,2). Recent observations clearly demonstrate that multiple types of T_r cells can be distinguished by lineage, expression of surface markers and cytokine production (1,3–5). A large number of recent studies have focused on naturally occurring $CD4^+CD25^+$ T_r cells, which are derived from the thymus, and, once activated via the TCR, can suppress systemic immune responses against self and foreign antigens. This was demonstrated by depletion of this T_r cell population in mice, which induces severe and fatal autoimmunity, characterized by wasting disease and autoimmune inflammation in multiple organs (6). Transfer of these $CD4^+CD25^+$ T_r cells to lymphopenic recipients prevents the development of autoimmunity (6–9). The mechanism of this suppression is dependent on cell–cell interactions, possibly mediated via cytotoxic T lymphocyte-associated antigen (CTLA)-4, and

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secretion of immunoregulatory cytokines such as IL-10 and transforming growth factor (TGF)-β (8,10,11).

In addition to these thymic-derived T_r cells, another subset of T_r cells is generated in the periphery in response to administration of soluble antigen via the mucosa (1). Recently, we observed that intrinsic characteristics of the nasal draining lymph nodes are critically important for the induction of tolerance via the nasal mucosa. A series of transplantation experiments revealed not only that the presence of cervical lymph nodes was necessary for nasal tolerance induction, but also that this function could not be assumed by peripheral lymph nodes when transplanted to this site (12). After nasal or oral tolerance induction, the T_r cells that can be isolated from the spleen and that are able to transfer tolerance, are $CD4^+$ T cells (mucosal T_r cells), but not $CD8^+$ T cells (13,14). More detailed knowledge of the phenotype of these mucosal T_r cells is lacking.

It is unclear what fate the transferred mucosal T_r cells undergo in the naive recipient. Do the transferred T_r cells expand by proliferation, establishing an exponentially increasing cohort of cells that suppress a subsequent sensitization and challenge? Or do these mucosal T_r cells transfer the regulatory capacity to a naive $CD4^+$ T cell of the recipient that subsequently suppresses sensitization and challenge?

Furthermore, little is known about the plasticity of the T_r cells that are generated after mucosal tolerance induction. During polarization of naive T cells into effector T_h1 and T_h2 cells, their plasticity is lost. As a result, after three to four cell divisions, T_h1 and T_h2 cells are incapable of re-expressing alternative cytokines when re-stimulated under opposing conditions (15). Previously, we have established that application of ovalbumin (OVA) via the mucosa is effective in the suppression of highly polarized T_h1 and T_h2 immune responses. It is unclear whether, after adoptive transfer and subsequent T_h1 or T_h2 sensitization and challenge, the mucosal T_r cells are influenced by the polarized environment in the host. Do the T_r cells retain their suppressive capacity in the recipient or do these cells acquire an irreversible differentiation analogous to T_h1 and T_h2 cells?

In this study we have addressed three questions on the fate, plasticity and identity of mucosal T_r cells that are generated after nasal tolerance induction. We show that T_r cells, induced by nasal OVA application, mediate tolerance by passing the tolerizing capacities on to naive $CD4^+$ T cells. Surprisingly, these mucosal T_r cells do not loose plasticity during their differentiation from naive $CD4^+$ T cells, as demonstrated by the capacity to suppress naive T cells irrespective of initial or ongoing cytokine polarization of the immune response. Finally, we show that T_r cells reside in both $CD25^+$ and $CD25^–$ subpopulations; however, only the $CD25^–$ T_r cells display antigen specificity.

Methods

Mice

Female BALB/c mice (8–12 weeks old) were obtained from Charles River (Sulzfeld, Germany) and kept under standard animal housing conditions. The congenic C57BL/6-Ly5.1 and C57BL/6-Ly5.2 mice were bred at our own facilities, and used at 8–12 weeks of age. The C57BL/6-Ly5.1 and C57BL/6-Ly5.2 mice express different allotypes of the CD45 (Ly5) antigen, which can be distinguished with mAb. Mice used in experiments were sex and age matched. All experiments were approved by The Animal Experiments Committee of the VUMC.

Induction of tolerance and subsequent antigen sensitization for delayed-type hypersensitivity (DTH) or allergic response

To induce tolerance, mice received 10 μl saline containing 100 μg OVA (type VII; Sigma, St Louis, MO) intranasally on each of 3 consecutive days. Control mice received 10 μl saline intranasally. Previously, we have described two models resulting in opposite cytokine-skewing, denoted as T_h1 (lacking IgE) and T_h2 [lacking IFN-γ (13)].

Mice were sensitized for a T_h1 response as previously described (13). In short, 25 μl incomplete Freund's adjuvant (IFA; Difco, Detroit, MI) mixed with 25 μl saline containing 100 μg OVA was injected s.c. in the tail base 1 day after the last intranasal OVA or saline administration. Five days later, a challenge was given by injecting 20 μl saline, which contained 10 μg OVA, in the auricles of both ears. Directly before challenge, the initial thickness of the ear was determined with an engineer's micrometer (Mitutoyo, Tokyo, Japan). DTH responses were expressed as the mean increase in ear thickness of both ears 24 h post-challenge, following subtraction of the mean ear thickness before challenge. In some experiments, mice were sensitized and challenged for a T_h1 response using hen egg lysozyme (HEL; Sigma). The same amounts of HEL were used for sensitization and challenge as were used for sensitization and challenge with OVA. In all experiments the ear thickness was measured in a blinded fashion.

Sensitization for a T_h2 response was performed as described previously (14). Briefly, on days 1 and 14 after the last intranasal OVA treatment, mice received an i.p. injection of 2 μg OVA dissolved in 100 μl saline mixed with 100 μl aluminum hydroxide rehydragel (AlOH; Reheis Chemical, Berkely Heights, NJ). Four days after the second injection, mice were challenged intragastrically with 200 μl saline containing 1 mg OVA. As a parameter for the T_h2 response, IgE levels were measured. Blood was collected via a small incision in the tail vein at $t = –2$, 0, 2, 4 and 6 days with regard to the intragastric challenge, and stored as serum at –80°C until IgE levels were measured.

Measurement of serum IgE by ELISA

Both total and OVA-specific IgE serum levels were determined by using a modified capture ELISA as described elsewhere (16). In brief, Falcon Microtest III plates (Becton Dickinson, San Diego, CA) were coated overnight at 4°C with rat anti-mouse IgE [EM95 (16)]. As a positive control, anti-TNP IgE-3 (PharMingen, San Diego, CA) for total IgE or pooled serum from multiple OVA-boosted mice for OVA-specific IgE was used. Total IgE was detected with biotinylated rat anti-mouse IgE (PharMingen, San Diego, CA). For detection of OVA-specific IgE, digoxigenin-coupled OVA [coupled as described by van Halteren *et al.* (16)] was used. Subsequently, for both total and OVA-specific IgE determination, either peroxidase-conjugated streptavidin (Dako, Glostrup,

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Denmark) or peroxidase-coupled sheep anti-digoxigenin Fab fragments (Boehringer, Mannheim, Germany) was added. Finally, the enzymatic reaction was developed by addition of *o*-phenylenediamine dihydrochloride (Sigma, St Louis, MO) in 0.1 M phosphate-citrate buffer, containing 0.015% hydrogen peroxide. Absorbance was measured at 490 nm and IgE concentrations were determined with reference to the standard curves.

Experiments with C57BL/6-Ly5.1 and C57BL/6-Ly5.2 mice

C57BL/6-Ly5.1$^+$ mice were tolerized and sensitized for a DTH response as described. Seven days after challenge, spleens from tolerant and naive control mice were collected. Single-cell suspensions were obtained by mincing the spleens and straining them through 100-μm gauze. Erythrocytes were depleted from this cell suspension by incubation in a lysis buffer (150 mM NH_4Cl and 1 mM $NaHCO_3$, pH 7.4) for 5 min on ice. For partial purification of $CD4^+$ T cells, the spleen cells were incubated for 30 min on ice with a B cell-specific rat mAb [clone 6B2, anti-B220; affinity purified from culture supernatant of hybridoma cells with Protein G–Sepharose (Pharmacia, Uppsala, Sweden)]. Next, the cells were washed and resuspended in HBSS (Biowhittaker, Verviers, Belgium) supplemented with 1% heat-inactivated FCS. Subsequently, sheep anti-rat IgG Dynabeads (Dynal, Oslo, Norway) were added in a 2:1 bead:cell ratio. After 30 min incubation on a rotator at 4°C, positively stained B cells were depleted using a magnetic particle concentrator (MPC; Dynal). The remaining cell population contained between 50 and 60% $CD4^+$ T cells, as determined by flow cytometry (FACStar; Becton Dickinson). Previously, we have shown that the contaminating $CD8^+$ T cells do not transfer tolerance (14). The enriched $CD4^+$ T cells were washed, resuspended in saline and 2–5 $\times$ 10^6 cells were transferred to naive C57BL/6-Ly5.2$^+$ acceptor mice by i.v. injection via the lateral tail vein. Upon transfer, the acceptor mice were sensitized for a T_h1 response. Seven days after challenge, the spleens were collected from these C57BL/6-Ly5.2$^+$ tolerant mice and partially purified $CD4^+$ T cells were isolated from single-cell suspensions. To determine whether the $CD4^+Ly5.2^+$ T cells had also become suppressive, highly purified $CD4^+$ T cells were used. These were prepared by incubation of the enriched $CD4^+$ T cell population with biotinylated anti-Ly5.1 antibody (clone A20; affinity purified from culture supernatant of hybridoma cells with Protein G–Sepharose and biotinylated according to the manufacturer's instruction), FITC-conjugated anti-Ly5.2 antibody (clone AL-1; affinity purified from culture supernatant of hybridoma cells with Protein G–Sepharose and labeled with FITC according to the manufacturer's instructions) and phycoerythrin (PE)-conjugated anti-CD4 antibody (GK1.5; PharMingen) for 30 min on ice. After washing, $CD4^+Ly5.2^+Ly5.1^-$ T cells were sorted by flow cytometry. The cells were washed, resuspended in saline and 1 $\times$ 10^6 $CD4^+$ T cells were transferred to naive acceptor mice by i.v. injection via the lateral tail vein.

Transfer experiments with BALB/c mice

For transfer of tolerance, donor BALB/c mice were tolerized and sensitized for a T_h1 response or a T_h2 response as described. Spleens of T_h1-tolerant mice were collected and pooled 7 days post-challenge, whereas spleens of T_h2-tolerant animals were collected at 4 days post-challenge. As a control, spleens from naive BALB/c mice were taken and partially purified $CD4^+$ T cells were isolated from single-cell suspensions as described above.

For transfer of purified $CD4^+CD25^+$ T cells and $CD4^+CD25^-$ T cells, the enriched $CD4^+$ T cells were stained with FITC-conjugated anti-CD4 antibody (clone GK1.5, affinity purified from culture supernatant of hybridoma cells with Protein G–Sepharose and FITC-labeled according to the manufacturer's instructions), PE-conjugated anti-CD25 antibody (clone PC61, anti-IL2Rα; PharMingen) and biotinylated anti-MHC class II antibody (clone M5/114, affinity purified from culture supernatant of hybridoma cells with Protein G–Sepharose) for 30 min on ice. After washing, $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells were sorted (FACSVantage; Becton Dickinson). The $CD4^+CD25^+$ T cell population was approximately 90% pure (the remaining 10% consisted of 6% $CD4^-CD25^-$ cells and 4% $CD4^+CD25^-$ cells) and the purity of the $CD4^+CD25^-$ cell population was ~98% as determined by re-analysis. After washing, the cells were resuspended in saline and 10^5 cells were transferred to naive acceptor mice by i.v. injection via the lateral tail vein.

When $CD4^+CD25^+$ T cells and $CD4^+CD25^-$ T cells were purified using MACS microbeads, the enriched $CD4^+$ T cells were incubated with biotinylated anti-CD25 antibody (clone PC61; PharMingen) for 15 min on ice, followed by anti-biotin immunomagnetic beads (MACS; Miltenyi Biotec, Bergisch Gladbach, Germany) for 15 min at 4°C. Magnetic separation was performed with an MS column according to the suggested protocol. The $CD4^+CD25^+$ T cell population was ~80% pure (the remaining 20% consisted of 14% $CD4^-CD25^-$ cells and 6% $CD4^+CD25^-$ cells) and the purity of the $CD4^+CD25^-$ cell population was ~86% (the remaining 14% consisted mainly of $CD4^-CD25^-$ cells) as determined by re-analysis. After washing, the cells were resuspended in saline and 10^5 cells were transferred to naive acceptor mice by i.v. injection via the lateral tail vein.

To determine whether dendritic cells (DC) were capable of transferring tolerance, purified splenic DC were used. DC were isolated from a suspension of spleen cells using anti-CD11c immunomagnetic beads (MACS) according to the manufacturer's instructions. This enriched DC population was then incubated with FITC-conjugated anti-MHC class II antibody (clone M5/114) and PE-conjugated anti-CD11c antibody (clone HL3; PharMingen) for 30 min on ice. Further purified $CD11c^+$ MHC class II^+ DC were obtained by flow sorting. After washing, cells were resuspended in saline and the numbers indicated were transferred to naive acceptor mice by i.v. injection via the lateral tail vein.

Proliferation assays

Lymph nodes were isolated from tolerant and non-tolerant mice 1 week after ear challenge. Single-cell suspensions were cultured with 0.5 mg/ml OVA in U-bottomed 96-well plates at 5 $\times$ 10^6 cells/ml in DMEM (Gibco, Life Technologies, Breda, The Netherlands) supplemented with 10% heat-inactivated FCS (Biowhittaker), 50 U/ml sodium penicillin G (Biowhittaker), 50 μg/ml streptomycin (Biowhittaker), 2 mM L-glutamine (Biowhittaker) and 50 μM β-mercaptoethanol (Merck,

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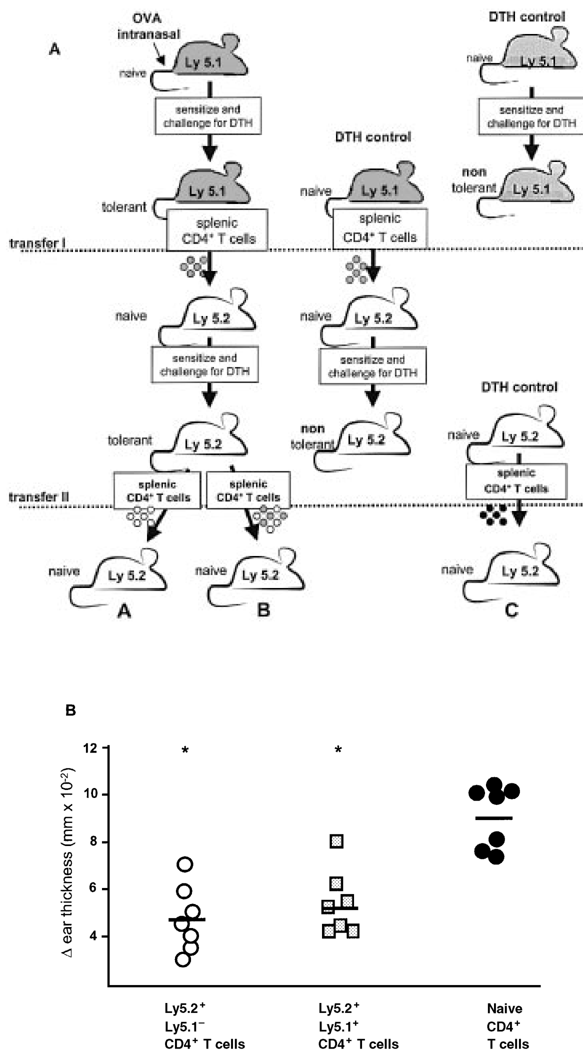

Fig. 1. Fate of T_r cells after transfer to a naive recipient. (A) C57BL/6-Ly5.1$^+$ mice were tolerized by intranasal instillation of 100 μg OVA on each of 3 consecutive days. One day later, mice were sensitized for a T_h1 response by a s.c. injection of 100 μg OVA in IFA, followed 5 days later by an injection of 10 μg OVA in each ear. Seven days after ear challenge $CD4^+$ splenic T cells from tolerant C57BL/6-Ly5.1$^+$ mice were isolated and 5×10^6 cells were adoptively transferred to naive C57BL/6-Ly5.2$^+$ mice by i.v. injection in the tail. Upon transfer, the C57BL/6-Ly5.2$^+$ acceptor mice were sensitized and challenged for a T_h1 response with OVA as described for the donor mice. Seven days after challenge, spleens were collected from the tolerant mice, and both $CD4^+$-enriched T cells (containing Ly5.1$^+$ as well as Ly5.2$^+$ T cells, grey squares) and highly purified $CD4^+$Ly5.2$^+$Ly5.1$^-$ T cells (open circles) were isolated and 1×10^6 cells were adoptively transferred to each of seven naive acceptor mice. (B) The acceptor mice of $CD4^+$-enriched T cells, the recipients of highly purified $CD4^+$Ly5.2$^+$ T cells and control mice which received 1×10^6 naive splenic $CD4^+$ T cells (black circles) were sensitized for a T_h1 response as previously described. Each point represents the mean increase in ear thickness of both ears per mouse 24 h after challenge of the final group of Ly5.1$^+$ acceptor mice. Bars indicate the mean of each group. An asterisk indicates a significant difference between acceptors of tolerant cells and control mice ($P < 0.001$). One representative experiment is shown. Similar results were also obtained in one further experiment ($n = 15$ per group).

Darmstadt, Germany). After 4 days, the cultures were pulsed with 0.8 μCi/well [^{3}H]thymidine (Amersham, Roozendaal, The Netherlands) and harvested 16 h later. Results are expressed as mean values of triplicates.

Statistics

For ear swelling responses, the mean increase in ear thickness of both ears was determined for each mouse per group. For T_h2 responses, data are expressed as mean IgE levels ± SD per group. Groups consist of seven mice, unless otherwise indicated. Groups were compared using an ANOVA followed by the Tukey–Kramer multiple comparisons test for ear swelling responses. For serum IgE levels, a MANOVA was used. For proliferation assays, data are expressed as mean values of triplicates. Groups were compared to the control group using an unpaired t-test. $P < 0.05$ was considered significant.

Results

Fate of T_r cells after transfer to a naive recipient

Application of OVA via the nasal mucosa leads to suppression of a subsequent DTH response, consisting of a sensitization with OVA in the tail base and a challenge in the ears. This tolerance can be transferred to naive recipients by i.v. injection of purified $CD4^+$ T_r cells from spleens of tolerant mice. It is unclear what the fate is of these $CD4^+$ T_r cells in the naive recipients. We hypothesized that the transferred $CD4^+$ T_r cells either expand by proliferation to develop an exponentially growing cohort of suppressor cells that suppress sensitization and challenge or, on the other hand, that these $CD4^+$ T_r cells, in the presence or absence of an antigen-presenting cell (APC), transfer their regulatory capacity to the naive $CD4^+$ T cells in the recipient.

To follow the fate of the tolerizing T_r cells, mice with an allelic difference in Ly5 (CD45) were used which enabled us to discriminate between donor and recipient cells. Donor C57BL/6-Ly5.1 mice received an intranasal OVA instillation on 3 consecutive days. One day after the last nasal intranasal OVA administration, mice were sensitized for a DTH response by an injection of OVA/IFA s.c. Five days later, mice were challenged by an injection of OVA in the auricles of both ears. Nasal OVA treatment led to tolerance as only marginal ear swelling was measured (mean increase in ear thickness was 5.25 ± 0.54 compared to 10.58 ± 2.75 x 10^{-2} mm in control mice treated nasally with saline). From these tolerant C57BL/6-Ly5.1 mice splenic $CD4^+$ T cells were purified and transferred into naive C57BL/6-Ly5.2 recipient mice (transfer I, Fig. 1A). These C57BL/6-Ly5.2 recipient mice were then sensitized and challenged with OVA to confirm that they had become tolerant after this cell transfer (mean increase in ear thickness was 5.43 ± 0.73 versus control 9.83 ± 1.66 x 10^{-2} mm). To rule out the possibility that a non-responsive T cell population was induced, simply due to the regimen of sensitization and challenge, splenic $CD4^+$ T cells from mice that had received saline intranasally were adoptively transferred to naive recipients. Transfer of $CD4^+$ T cells from these OVA-primed mice did not inhibit the DTH response in the recipient mice (mean increase in ear thickness was 10.88 ± 1.62 versus control

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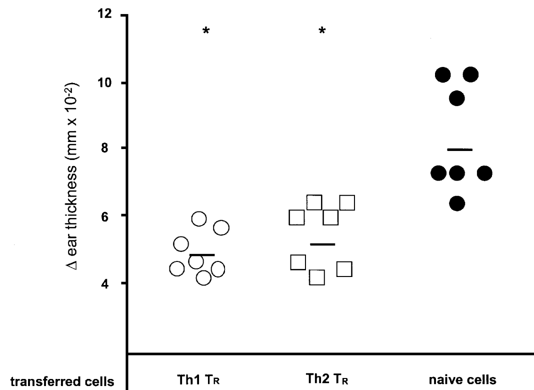

Fig. 2. $CD4^+$ T_r cells derived from a strongly T_h2-biased donor retain their plasticity to suppress a T_h1 response. BALB/c mice were treated intranasally with 100 μg OVA on each of 3 consecutive days. One day later, half of the mice were sensitized for a T_h1 response by a s.c. injection of 100 μg OVA in IFA, followed 5 days later by an injection of 10 μg OVA in both ears. The second half of the group was sensitized for a T_h2 response by two i.p. injections of 2 μg OVA in AlOH with a 2-week interval, followed by an intragastric injection of 1 mg OVA 4 days after the second i.p. injection, according to van Halteren *et al.* (18). $CD4^+$ T cells (5×10^6) from T_h1-tolerant mice were transferred 7 days after challenge to naive mice (T_h1 acceptors). T cells (5×10^6) from T_h2-tolerant mice were transferred at 4 days after challenge (T_h2 acceptors). Control mice received 5×10^6 naive splenic $CD4^+$ T cells. Upon transfer, seven mice in each of the T_h1 acceptor, T_h2 acceptor and control groups were sensitized for a T_h1 response. Each point represents the mean increase in ear thickness of both ears per mouse 24 h after challenge with OVA. Bars represent the mean of each group. An asterisk indicates a significant difference between acceptors of T_h1- or T_h2-tolerant cells and control mice ($P < 0.0003$). Similar results were obtained in one further experiment.

$11.81 \pm 2.49 \times 10^{-2}$ mm), indicating that these cells remain responsive to antigenic stimulation.

This adoptive transfer system was then pursued further to determine whether the original donor $Ly5.1^+CD4^+$ T_r cells were responsible for the suppression in the recipient mice or whether these T_r cells had transferred their regulatory capacity on to the $CD4^+$ T cells of the C57BL/6-Ly5.2 recipient. Therefore, splenic cells of the tolerant recipients were stained for donor (Ly5.1) and recipient (Ly5.2) allotypes, and separated into a purified population of $Ly5.2^+Ly5.1^-CD4^+$ T cells (recipient), and a population containing both $Ly5.1^+$ (donor) and $Ly5.2^+CD4^+$ (recipient) T cells. The $Ly5.2^+CD4^+$ T cell population was ~97% pure as determined by flow cytometric re-analysis. The contaminating 3% were $Ly5.2^+CD4^-$ cells, but contained no Ly5.1 donor cells. Subsequently, 1×10^6 cells of each population were transferred to naive acceptor mice in a second series of transfers (transfer II, Fig. 1A). As a control, 1×10^6 splenic $CD4^+$ T cells from naive, non-OVA-exposed mice were also transferred to naive recipients.

As shown in Fig. 1(B), the $Ly5.2^+CD4^+$ T cells of the recipient of transfer I are able to suppress the DTH response in naive recipients (group A), indicating that they have become T_r cells. Recipients of both $Ly5.1^+$ and $Ly5.2^+CD4^+$ T cells (group B) were also tolerant, as ear swelling responses were similar to those in recipients of $Ly5.2^+CD4^+$ T cells, but significantly different from recipients of naive $CD4^+$ T cells (group C). Moreover, it should be noted that at the time of isolation of $CD4^+$ cells for transfer II, the spleens of the C57BL/6-$Ly5.2^+$ mice contained only a minor fraction of $CD4^+$ T cells (<0.5% of total $CD4^+$ T cells) of the original donor Ly5.1 allotype.

Recent studies have shown that APC can mediate T cell suppression, either upon cross talk with 'anergic' T cells, or via the secretion of IL-10 and/or TGF-β (17,18). To exclude the possibility that the results obtained from both T cell transfers I and II were due to contaminating DC, adoptive transfers were performed with purified splenic DC from OVA-tolerized mice. In our hands, transfer of 5×10^4 DC, a number which is at least 5-fold excess of the possibly contaminating DC in both transferred T cell populations, did not inhibit the DTH response in recipient mice (mean increase in ear thickness was 8.45 ± 1.15 versus control $9.56 \pm 1.15 \times 10^{-2}$ mm).

Together, these results clearly demonstrate that T_r cells, when transferred to naive recipients, induce the $CD4^+$ T cells of the recipient to differentiate into T_r cells.

Transferred $CD4^+$ T_r cells retain their suppressive capacity in a polarized environment

In the experiments described above, mice were challenged to develop a DTH response, a type of stimulus that is biased towards inducing T_h1 cells. Previously, we have shown in an allergy model that nasal OVA application can effectively tolerize polarized T_h2 responses, as measured by the suppressive effect on both total and OVA-specific IgE production (16). Since upon differentiation T_h1 and T_h2 cells undergo irreversible lineage commitment, we questioned whether after defined sensitization and challenge T_r cells would also lose their plasticity. To test this, we assessed whether T_r cells from a T_h1-biased animal would be able to suppress the sensitization of both T_h1 and T_h2 responses, and whether this also applied to T_r cells from a T_h2-biased animal.

To induce tolerance, mice received a nasal OVA application on each of 3 consecutive days. Thereafter, mice were divided in two groups. One group was sensitized for a DTH response by an injection of OVA/IFA s.c. (T_h1-type response). Five days later, mice were challenged by an injection of OVA in the auricles of both ears. It was confirmed that nasal OVA-treated mice were tolerant (mean increase in ear thickness was $4.03 \pm 0.66 \times 10^{-2}$ mm), as ear swelling responses were reduced in comparison to saline-treated mice, which displayed significantly enhanced ear swelling responses (mean increase in ear thickness was $9.38 \pm 1.27 \times 10^{-2}$ mm). The other group was sensitized for an allergic response by two i.p. injections of OVA in AlOH with a 2-week interval (T_h2-type response). Four days after the last i.p. injection, mice were challenged for IgE by an intragastric injection of OVA. In the sera from mice treated with OVA intranasally significantly lower IgE levels were detected (2687.99 ± 753.64 ng/ml total IgE) than in sera from saline treated mice (8805.25 ± 588.32 ng/ml total IgE, on day 4 after challenge), indicating tolerance. From the spleens from T_h1-tolerant and T_h2-tolerant mice, $CD4^+$ T_r cells were purified and transferred into naive recipients (T_h1 and T_h2 acceptors respectively). Control mice received naive splenic $CD4^+$ T cells. Upon transfer, T_h1 acceptors, T_h2 acceptors and control mice were divided in two groups; half of the mice were sensitized for a T_h1 response, whereas the other half of the mice were sensitized for a T_h2 response. As depicted in Fig. 2,

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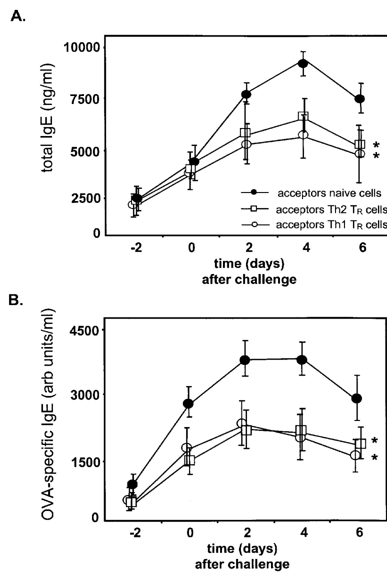

Fig. 3. CD4+ T_r cells derived from a strongly T_h1-biased donor retain their plasticity to suppress a T_h2 response. BALB/c mice were treated intranasally with 100 µg OVA on each of 3 consecutive days. One day later half of the mice were sensitized for a T_h1 response, whereas the second half were sensitized for a T_h2 response, as described in the legend to Fig. 2. CD4+ T cells (5×10^6) from either T_h1-tolerant or T_h2-tolerant mice were transferred to naive mice (T_h1 acceptors and T_h2 acceptors respectively). Control mice received 5×10^6 naive splenic CD4+ T cells. Upon transfer, the T_h1 acceptors, T_h2 acceptors and control mice were sensitized for a T_h2 response. The mean level of total (A) and OVA-specific (B) IgE of seven mice is shown. An asterisk indicates a significant difference between acceptors of T_h1- or T_h2-tolerant cells and control mice ($P < 0.0003$). Similar results were obtained in one further experiment.

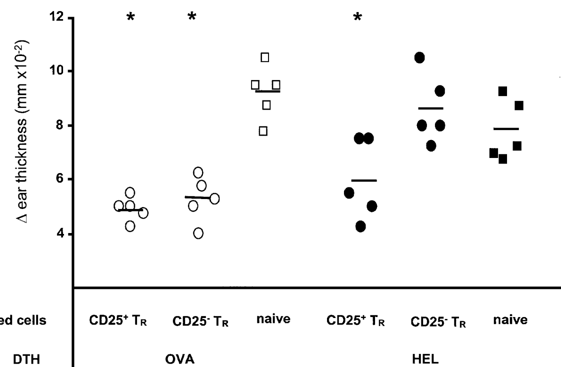

Fig. 4. Suppression by the CD4+CD25– T cell subset is specific for the antigen used for intranasal tolerization, whereas that of CD4+CD25+ T cells is not. Mice were treated intranasally with 100 µg OVA on each of 3 consecutive days followed by sensitization for a DTH response as described in the legend to Fig. 1. One week later, CD4+CD25+ T cells and CD4+CD25– T cells were isolated from the spleens of these tolerant mice, and adoptively transferred to groups containing five naive acceptor mice. Subsequently, the recipients of 10^5 highly purified CD4+CD25+ or CD4+CD25– T cells were sensitized for a DTH response, as described in the legend to Fig. 1, either with OVA (open symbols) or HEL (black symbols). Control mice received 10^5 naive splenic CD4+ T cells. Each point represents the mean increase in ear thickness of both ears per mouse 24 h after challenge. Bars indicate the mean of each group. An asterisk indicates a significant difference between acceptors of T_r cells and control mice ($P < 0.01$). Similar results were obtained in two additional experiments.

the acceptor mice that were sensitized for a T_h1 response showed a similar reduction in DTH response irrespective whether the transferred CD4+ T_r cells were derived from T_h1- or T_h2-tolerant animals.

Similar results were found when acceptor mice were sensitized for a T_h2 response (Fig. 3A and B). Where control mice developed substantial (total and anti-OVA) IgE levels, strongly reduced IgE levels were seen in animals that had received either T_h1 or T_h2 T_r cells prior to sensitization and challenge (Fig. 3A and B).

In conclusion, these results clearly show that CD4+ T_r cells derived from a strongly T_h2-biased donor retain their capacity to suppress a T_h1 response and *vice versa*.

Identification of subsets of mucosal CD4+ T_r cells

Until now, it has been unclear whether the regulatory activity of mucosal T_r cells is limited to a specific subset of CD4+ splenocytes. Flow cytometric analysis of CD4+ T_r splenocytes and control CD4+ splenocytes from a non-tolerized donor did not yield any significant phenotypic differences. Since the detection limit may be too high to distinguish a discriminating marker of a T_r subset and in view of the vast amount of data demonstrating a role for CD4+CD25+ T cells in immune regulation (5), we determined whether the mucosal CD4+ T_r cells are CD25+ or CD25–. Therefore, mice were tolerized by a nasal OVA instillation on 3 consecutive days. These mice were then sensitized for a T_h1 response by an injection of OVA/IFA s.c. and challenged by an injection of OVA in the auricles of both ears to confirm tolerance (mean increase in ear thickness was 4.7 ± 0.48 versus control $9.56 \pm 0.72 \times 10^{-2}$ mm).

One week after challenge, splenic CD4+ T_r cells from the T_h1-tolerant mice were sorted into CD25+ (90% pure) and CD25– (98% pure) fractions, and subsequently transferred to naive acceptor mice. As a control, splenic CD4+ T cells were from naive, non-OVA-exposed mice were also transferred to naive recipients. The acceptor mice were then sensitized and challenged for a T_h1 response with either OVA or an unrelated antigen HEL. It was observed that the CD4+CD25+ T cells from tolerant mice were able to suppress both the OVA- and HEL-specific DTH responses, whereas the CD4+ T cells from non-tolerized donors failed to induce tolerance to either antigen in the recipient (Fig. 4). In contrast to the CD4+CD25+ subset, the CD4+CD25– T cells only suppressed the DTH response against OVA, but not the DTH response against HEL. This demonstrates that suppression by the CD4+CD25– population requires the antigen used for tolerization, whereas suppression by the CD4+CD25+ T cells is not specific for the applied antigen.

To confirm the *in vivo* data shown in Fig. 4, facial lymph node cells of the recipient mice were isolated after sensitization and challenge with either OVA or HEL, and proliferative responses were measured *in vitro*. As shown in Fig. 5, T cell proliferative responses to OVA were suppressed in cultures of CD4+CD25+ T cell recipients as well as CD4+CD25– T cell recipients when

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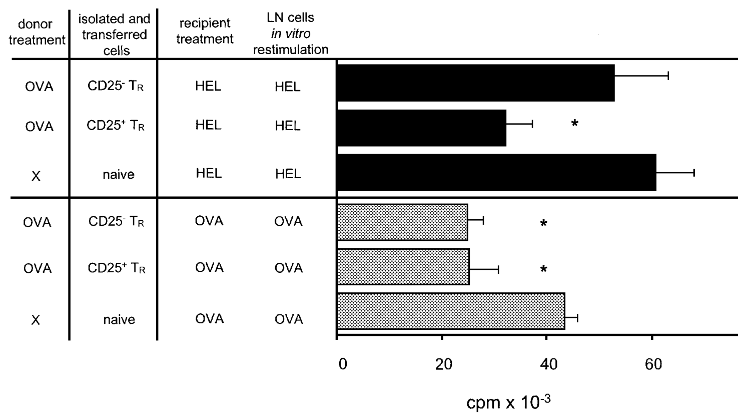

Fig. 5. Proliferative OVA-tolerized T cell responses to HEL are only suppressed in lymph node cultures of $CD4^+CD25^-$ T cell acceptors. Mice were treated intranasally with 100 μg OVA on each of 3 consecutive days followed by sensitization for a DTH response as described in the legend to Fig. 1. One week later, $CD4^+CD25^+$ T cells and $CD4^+CD25^-$ T cells were isolated from the spleens of tolerant mice, and adoptively transferred to groups of seven naive acceptor mice. Subsequently, the recipients of 10^5 highly purified $CD4^+CD25^+$ or $CD4^+CD25^-$ T cells were sensitized for a T_h1 response either with OVA or the unrelated antigen HEL. Control mice received 10^5 naive splenic $CD4^+$ T cells. Seven days after ear challenge, facial lymph nodes were collected and re-stimulated *in vitro*. Four days later cultures were pulsed with [^{3}H]thymidine and incorporation was determined. An asterisk indicates a significant difference between acceptors from T_r cells and controls ($P < 0.05$).

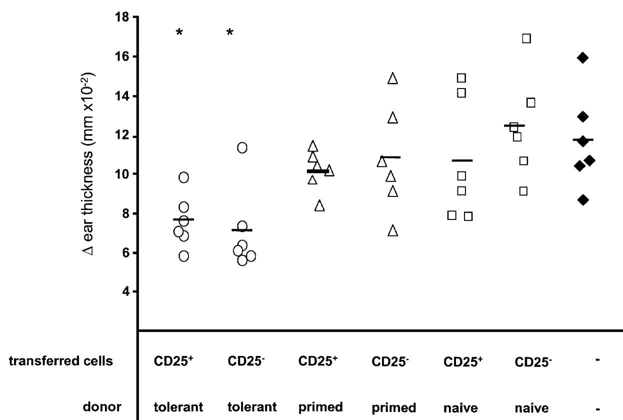

Fig. 6. The $CD4^+CD25^+$ T cell subset from OVA-tolerized mice is induced by mucosal OVA application. Mice were treated intranasally either with 100 μg OVA or saline on each of 3 consecutive days followed by sensitization for a DTH response as described in the legend to Fig. 1. One week later, $CD4^+CD25^+$ T cells and $CD4^+CD25^-$ T cells were purified from the spleens of tolerant, primed and naive mice by MACS microbeads, and adoptively transferred to groups of six naive acceptor mice. Subsequently, the recipients of 10^5 highly purified $CD4^+CD25^+$ or $CD4^+CD25^-$ T cells were sensitized for a T_h1 response as described in the legend to Fig. 1. Control mice had not received splenic $CD4^+$ T cells. Each point represents the mean increase in ear thickness of both ears per mouse 24 h after challenge. Bars indicate the mean of each group. An asterisk indicates a significant difference between the control group (mice that had not received T cells) and all other groups ($P < 0.05$).

compared to cells from naive T cell recipients. In contrast, proliferative responses to HEL were only suppressed in cultures of $CD4^+CD25^+$ T cell recipients, whereas the responses of $CD4^+CD25^-$ T cell recipients and naive T cell recipients were comparable. Thus, these data mirror and confirm the results obtained *in vivo*.

Since the $CD25^+$ T_r cells do not show antigen specificity and in this respect resemble the thymic-derived $CD25^+$ T_r cells, we wondered whether the latter contributed to the observed suppression. Therefore, adoptive transfers were performed with purified $CD4^+CD25^+$ T cells and $CD4^+CD25^-$ T cells from tolerized, primed and naive mice. Consequently, mice that had received either OVA or saline intranasally on 3 consecutive days were sensitized for a T_h1 response by an injection of OVA/IFA s.c. and challenged by an injection of OVA in the auricles of both ears. It was confirmed that nasal OVA-treated mice were tolerant (mean increase in ear thickness was $3.95 \pm 1.09 \times 10^{-2}$ mm), as ear swelling responses were reduced in comparison to saline-treated mice, which displayed enhanced ear swelling responses (mean increase in ear thickness was $8.95 \pm 1.24 \times 10^{-2}$ mm). One week after challenge, splenic $CD4^+$ T cells from tolerant, non-tolerant (primed) and naive mice were purified into $CD25^+$ (80% pure) and $CD25^-$ (87% pure) fractions, and subsequently transferred to naive acceptor mice. The acceptor mice were then sensitized and challenged for a T_h1 response with OVA. As a control, mice that had not received T cells were sensitized and challenged for a T_h1 response with OVA. As depicted in Fig. 6, DTH responses were significantly suppressed in mice that had received $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells from tolerized mice, whereas no suppression was observed in those that received the same subsets from naive or from primed mice as ear swelling was as pronounced as seen in control mice.

In conclusion, the results clearly demonstrate that mucosal T_r cells are present in both $CD4^+CD25^+$ and $CD4^+CD25^-$ subsets. Moreover, only in the $CD4^+CD25^-$ subset, but not in the $CD4^+CD25^+$ T cell subset, were T_r cells found that were specific for the antigen to which tolerance was generated.

Discussion

In this report we have studied fate, plasticity and the identity of mucosal T_r cells in a murine model of adoptive transfer.

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We report that in mice, application of OVA via the nasal mucosa induces $CD4^+$ T_r cells in the spleen that mediate tolerance by transferring their regulatory capacity to naive T cells *in vivo*. These T_r cells retain their plasticity in polarized microenvironments, as demonstrated by the finding that mucosal T_r cells recovered from a T_h2-biased mouse are capable of suppressing both T_h1 and T_h2 responses, and with equal potency as mucosal T_r cells recovered from a T_h1-biased mouse. Moreover, both $CD4^+CD25^+$and $CD4^+$ $CD25^-$ subsets exerted regulatory activity. However, only the $CD4^+CD25^-$ suppressed responses specific for the antigen used for tolerization.

Our observation that mucosal T_r cells transfer their regulatory activity to naive T cells in a naive recipient resembles properties of T_r cells that were described in tissue transplantation studies (19). In these studies, tolerance induced to skin grafts using non-depleting anti-T cell mAb was reported as being 'infectious'. It is unclear why mucosal T_r cells transfer their function instead of expanding exponentially. However, the characteristic of poor division has been observed for many cells with suppressor activity. For example, naturally existing $CD25^+$ T_r have been reported to be anergic to stimulation via the TCR, but to proliferate and even lose their tolerogenic capacity upon addition of exogenous IL-2 (20,21).

The cellular interactions that regulate the transfer of the suppressive activity from a T_r cell to a naive T cell may comprise a sequence of events. Initially, after i.v. transfer and subsequent sensitization of the acceptor mouse with OVA, the T_r cell may encounter an APC that presents OVA in the vicinity of a naive $CD4^+$ T cell. Upon recognition of antigen in the context of an APC the T_r cell will be activated and may regulate the naive T cell via multiple pathways. Regulation may involve direct signaling, e.g. via Notch, that has previously been suggested to block the differentiation of precursor T cells into both T_h1 and T_h2 cells (22). Furthermore, since T cells also express Notch ligands, the suppressed T cells may signal to adjacent naive T cells to further spread the block in differentiation and may thus render cells competent for subsequent regulation steps (23). Other regulatory mechanisms may be provided by the secretion of anti-inflammatory cytokines, such as IL-10 and TGF-β by either the T_r cell or the APC (10,11,18,24). However, it has been shown that IL-10 does not play a role in nasal tolerance, since administration of anti-IL-10 mAb either during the induction or during sensitization does not restore DTH responses in nasally tolerized mice (13). A recent *in vitro* study using human naturally existing $CD25^+CD4^+$ T_r cells elegantly showed that these T_r cells need cell–cell interaction for suppression and transfer of tolerogenic potencies, whereas the induced secondary $CD4^+$ suppressor T cells do not (25). Instead, these cells inhibit via soluble mediators like TGF-β. Whether these mechanisms are also operating in our *in vivo* model is unclear. However, the $CD25^-$ T_r subset may suppress differently than the $CD25^+$ T_r population, since we observed that the former is antigen specific, whereas the latter is not. In addition, down-regulation of the response may involve surface molecules such as CTLA-4 and PD-1 (8,26). The contribution of each of these pathways is the subject of current investigation.

Surprisingly, the microenvironment in which the mucosal T_r cells spread their tolerance to naive cells does not affect the plasticity of the response. The potency to suppress T_h1 and T_h2 responses did not differ between T_r cells derived from a T_h1-biased mouse and a T_h2-biased mouse. Although these data suggest that T_r cells retain their plasticity, it cannot be excluded that within the T_r population 'T_h1- or T_h2-biased' T_r cells exist. This broad down-regulatory activity is very encouraging when we consider the potential use of mucosal T_r cell induction for the treatment of immunological disorders that are characterized by highly polarized cytokine profiles. Furthermore, since previous and ongoing cytokine responses do not alter the suppressive capacity of the T_r cell, this observation indirectly underlines the tight regulation with which the T_r cell exerts its suppressive function.

To unravel the intrinsic properties of mucosal T_r cells, it is of key importance to determine their phenotype and to assess their relationship with other types of T_r cells that have been described. In this study, we demonstrate that both splenic $CD4^+CD25^+$ and $CD4^+CD25^-$ T cell subsets isolated from mice that were tolerized intranasally with OVA were equally capable of suppressing a systemic challenge to OVA. However, only the $CD4^+CD25^-$ T cell subset shows antigen specificity.

Our observation that the $CD4^+CD25^+$ T cell population suppresses responses to the unrelated antigen HEL is in accordance with the current literature and suggests that these cells may represent a peripheral version of the thymus-derived $CD4^+CD25^+$ T_r cells (6,27). However, if the $CD4^+CD25^+$ T_r subset would largely be constituted of thymus-derived T_r cells, it could be expected that the $CD4^+CD25^+$ subset from naive mice would also suppress the DTH response. Since the latter subset failed to induce tolerance in the recipient mice at the same ratios, it may imply that the $CD4^+CD25^+$ T_r cells from nasally tolerized mice are indeed mucosally induced, as has been suggested for low-dose oral tolerance induction (28,29). The absence of suppressive activity by the $CD25^+$ T_r subset from naive mice could be explained by the fact that it may depend on the ratio of suppressor and effector cells.

We are the first to report that $CD4^+CD25^-$ T cells induced by nasal antigen application suppress systemic challenge in an antigen-specific manner. It is questioned where these T cells are generated. We have previously shown that the cervical lymph nodes are essential for the induction of nasal tolerance (12) and therefore we hypothesize that the application of OVA via the nasal mucosa causes naive T cells to differentiate into $CD4^+CD25^-$ T_r cells under the influence of a specific microenvironment present only in these lymph nodes. Moreover, once mucosal T_r cells have been generated, other naive $CD4^+$ T cells may acquire their suppressive capacity in non-mucosal tissue via the mechanism of 'infectious' tolerance. Whether the $CD4^+CD25^-$ T cells are related to the recently described naturally existing $CD4^+CD25^+$ T_r subset is unclear. To our knowledge, there is no evidence that the thymus exports any $CD4^+$ T cells pre-committed to immune regulation within the $CD4^+CD25^-$ subset (30).

In sum, we report that in nasal tolerance, T_r cells are operating that transfer their tolerogenic potencies to naive T cells. Moreover, we identified a $CD4^+CD25^-$ T_r subset that is antigen specific, but exerts its suppressive function regardless of initial or ongoing cytokine polarization.

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Abbreviations

APC	antigen-presenting cell
CTLA	cytotoxic T lymphocyte-associated antigen
DC	dendritic cell
DTH	delayed-type hypersensitivity
IFA	incomplete Freund's adjuvant
HEL	hen egg lysozyme
OVA	ovalbumin
PE	phycoerythrin
TGF	transforming growth factor

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