

Differential Regulatory Function of Resting and Preactivated Allergen-Specific CD4⁺CD25⁺ Regulatory T Cells in Th2-Type Airway Inflammation¹

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Although CD4⁺CD25⁺ regulatory T (Treg) cells are known to suppress Th1 cell-mediated immune responses, their effect on Th2-type immune responses remains unclear. In this study we examined the role of Treg cells in Th2-type airway inflammation in mice. Depletion and reconstitution experiments demonstrated that the Treg cells of naive mice effectively suppressed the initiation and development of Th2-driven airway inflammation. Despite effective suppression of Th2-type airway inflammation in naive mice, adoptively transferred, allergen-specific Treg cells were unable to suppress airway inflammation in allergen-prensensitized mice. Preactivated allergen-specific Treg cells, however, could suppress airway inflammation even in allergen-prensensitized mice by accumulating in the lung, where they reduced the accumulation and proliferation of Th2 cells. Upon activation, allergen-specific Treg cells up-regulated CCR4, exhibited enhanced chemotactic responses to CCR4 ligands, and suppressed the proliferation of and cytokine production by polarized Th2 cells. Collectively, these results demonstrated that Treg cells are capable of suppressing Th2-driven airway inflammation even in allergen-prensensitized mice in a manner dependent on their efficient migration into the inflammatory site and their regulation of Th2 cell activation and proliferation. *The Journal of Immunology*, 2008, 181: 6889–6897.

Allergic asthma is a chronic airway inflammatory disorder characterized by airway hyperresponsiveness (AHR)³ and reversible airway obstruction, eventually leading to airway remodeling and irreversible changes in lung function (1). Although eosinophils are the predominant infiltrating cell type in the lungs of asthmatic subjects, Th2 cells also play a critical role in the pathogenesis of asthma via secretion of cytokines, including IL-4, IL-5, IL-9, and IL-13; IL-4 and IL-5 contribute to eosinophil accumulation, whereas IL-9 and IL-13 induce AHR (2).

Naturally occurring CD4⁺CD25⁺ regulatory T (Treg) cells of thymic origin play a significant role in maintaining peripheral self-tolerance by preventing the activation and proliferation of autoreactive T cells (3). CD4⁺CD25⁺ Treg cells also regulate immune responses against foreign Ags, including infectious agents and alloantigens, via suppression of Ag-specific CD4⁺ and CD8⁺ T cells (4–7). Although the suppression of Th1-mediated diseases by CD4⁺CD25⁺ Treg cells has been well documented (8, 9), the role of these cells in curtailing allergic Th2-mediated diseases is still controversial. Although human studies suggest that CD4⁺CD25⁺ Treg cells also suppress Th2-type diseases (10–12), mouse studies have yielded contradictory results. Suto et al. demonstrated that CD4⁺CD25⁺ T cells enhanced Th2 cell-mediated allergic inflammation in airways by modulating the Th1/Th2 balance toward a Th2 phenotype (13). In contrast, Jaffar et al. reported that CD4⁺CD25⁺ T cells curtailed Th2-mediated pulmonary inflammation by suppressing development of the Th2 phenotype (14). Adoptive transfer of CD4⁺CD25⁺ T cells into allergen-sensitized mice or into naive mice with cotransfer of allergen-specific Th2 cells has also presented inconsistent results, whereas CD4⁺CD25⁺ Treg cells reverse airway inflammation in some, but not all, cases (14–16). The available data do not permit a definitive conclusion regarding the role of CD4⁺CD25⁺ Treg cells in Th2-type airway inflammation or, more importantly, the rationale for use in the treatment of allergic asthmatics.

In this study, we demonstrated that CD4⁺CD25⁺ Treg cells were capable of suppressing airway inflammation in naive mice. Depletion of CD25⁺ cells enhanced airway inflammation and increased eosinophil numbers in the lung. Reconstitution with CD4⁺CD25⁺ Treg cells before allergen sensitization/challenge reduced these effects. We revealed that allergen-specific CD4⁺CD25⁺

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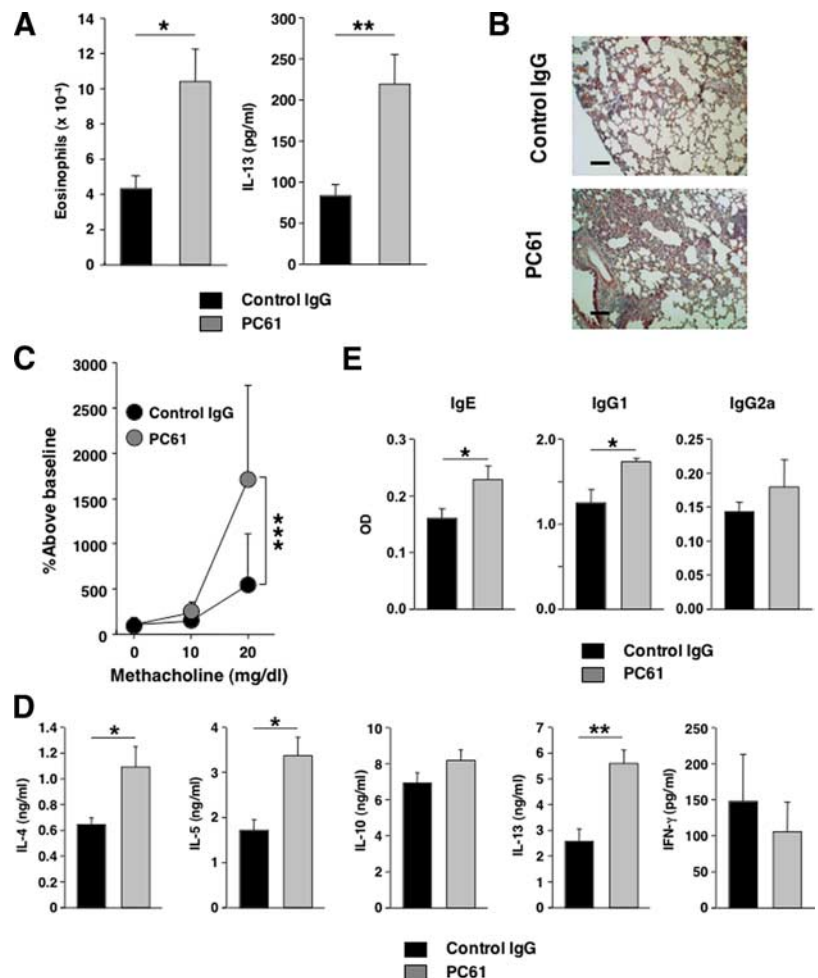
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³ Abbreviations used in this paper: AHR, airway hyperresponsiveness; BALF, bronchoalveolar lavage fluid; BLN, bronchial lymph node; GPCR, glucocorticoid-induced TNFR-related protein; i.n., intranasal(ly); PD-L1, programmed death ligand 1; TARC, thymus- and activation-regulated cytokine; Treg, regulatory T.

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FIGURE 1. Mice depleted of CD25⁺ cells developed severe airway inflammation and AHR. BALB/c mice ($n = 5-7$) were injected i.v. with anti-CD25 mAb (PC61; 250 μ g) or control rat IgG, then sensitized and challenged with OVA. Twenty-four hours after the last challenge, BALF, lung, BLN, and serum samples were collected or AHR was measured. **A**, Depletion of CD25⁺ cells resulted in increased numbers of eosinophils and IL-13 levels in BALF. **B**, H&E staining revealed increased numbers of eosinophils in the lungs of mice depleted of CD25⁺ cells. Representative sections for each group are shown. Bars, 100 μ m. **C**, CD25⁺ cell depletion increased AHR in response to methacholine. **D**, Depletion of CD25⁺ cells increased Th2-type cytokine production by BLN cells stimulated in vitro with OVA. **E**, CD25⁺ cell depletion increased the serum levels of OVA-specific Th2-type Ig isotypes. The serum levels of OVA-specific IgE, IgG1, and IgG2a were assayed at 1/300, 1/3 $\times 10^5$, and 1/1000 dilutions, respectively. These results are representative of three (**A** and **B**) and two (**C-E**) independent experiments. In **A** and **C-E**, the results are expressed as the means $\pm$ SEM between animals ($n = 5-7$); *, $p < 0.05$, **, $p < 0.01$; ***, $p < 0.001$.



Treg cells, if preactivated before adoptive transfer, efficiently suppressed the airway inflammation induced in allergen-sensitized mice. Upon activation, allergen-specific CD4⁺CD25⁺ Treg cells up-regulated CCR4, which lead to the enhancement of chemotactic responses in response to the associated ligands. After adoptive transfer, these cells efficiently accumulated in the lung, where CCR4 ligands are highly expressed (17, 18).

Materials and Methods

Mice

Female BALB/c and C.B-17 SCID mice were purchased from Japan SLC and CLEA Japan, respectively. DO11.10 mice, which bear an $\alpha\beta$ TCR transgene that recognizes OVA₃₂₃₋₃₃₉ in the context of I-A^d, were provided by Dr. K. M. Murphy (Washington University, St. Louis, MO). Mice housed under specific pathogen-free conditions in the Institute of Laboratory Animals were used at 6–8 wk of age. All experiments were approved by the Ethics Committee for Animal Experimentation at Mie University Graduate School of Medicine, Tsu, Japan.

Cells

A CD4⁺CD25⁺ regulatory T cell isolation kit (Miltenyi Biotec) was used to separate CD4⁺CD25⁺ and CD4⁺CD25⁺ T cells from the spleens of BALB/c or DO11.10 mice. When indicated, CD4⁺ T cells were isolated using a CD4⁺ T cell isolation kit (Miltenyi Biotec) from bronchial lymph nodes (BLN) of BALB/c mice that had been sensitized and challenged with OVA. Prepared T cell preparations were >95% CD4⁺CD25⁺ cells, >90% CD4⁺CD25⁺ cells, and >95% CD4⁺ cells as assessed by flow cytometry. In selected experiments, negatively isolated CD4⁺ T cells from spleen cells of DO11.10 mice were sorted into CD4⁺CD25⁺ T cells by using a FACSARIA cell sorter (BD Biosciences) after staining with PerCP-Cy5.5-conjugated anti-CD4 and allophycocyanin-conjugated anti-CD25. The

sorted CD4⁺CD25⁺ T cells contained >98% CD4⁺CD25⁺ cells. OVA-specific Th2-polarized cells were generated in vitro using CD4⁺CD25⁺ T cells from DO11.10 mice. Briefly, DO11.10-derived CD4⁺CD25⁺ T cells (1×10^5 cells/ml) were cultured with 35 Gy-irradiated BALB/c spleen cells (2×10^6 cells/ml) in the presence of OVA₃₂₃₋₃₃₉ (1 μ g/ml) and IL-4 (10 ng/ml; Peprotech) and anti-IL-12p40 (C17.8; 5 μ g/ml; BD Pharmingen). Th2-polarized cells were maintained in IL-2 (2.5 ng/ml; Ajinomoto). Th2 polarization was confirmed by intracellular staining for IL-4 and IFN- γ after restimulation for 24 h with OVA₃₂₃₋₃₃₉ in the presence of splenic APC. Upon restimulation, >30% and <0.9% of the Th2-polarized cells expressed IL-4 and IFN- γ , respectively (data not shown). When indicated, T cell preparations were labeled with 1.25 μ M CFSE (Invitrogen) in PBS for 7 min at 37°C.

Preactivation of OVA-specific Treg cells

Purified CD4⁺CD25⁺ T cells isolated from DO11.10 mice were cultured for 48 h in wells coated with anti-CD3 (145-2C11; 2 μ g/ml) and anti-CD28 (37.51; 2 μ g/ml) Abs in the presence of IL-2 (recombinant human IL-2; 2.5 ng/ml, Ajinomoto).

In vitro assessment of regulatory activity

A fixed number of responder CD4⁺ T cell preparations (5×10^4 cells) originating from DO11.10 mice were cocultured for 72 h with graded numbers of resting or preactivated Treg cells in the presence of 35 Gy-irradiated BALB/c spleen cells (2×10^5 cells) and 1 μ g/ml OVA₃₂₃₋₃₃₉ peptide in a total volume of 250 μ l/well in 96-well round-bottom plates. The proliferative responses of the responder CD4⁺ T cells were measured by [³H]TdR incorporation or reductions in CFSE staining within the CD4⁺KJ1-26⁺ subset. When indicated, aliquots of the culture supernatants were collected after 72 h of culture and assayed for cytokine production by ELISA.

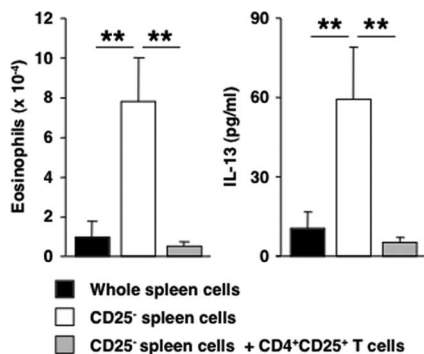


FIGURE 2. Regulatory role of CD4⁺CD25⁺ Treg in the development of airway inflammation. The immune systems of five C.B-17 SCID mice were reconstituted with total spleen cells (1×10^6) or CD25⁻ spleen cells (1×10^6) with or without CD4⁺CD25⁺ T cells (2×10^5) purified from BALB/c mice. Animals were then subjected to sensitization and challenge with OVA. Twenty-four hours after the last challenge, BALF was collected. Transfer of CD4⁺CD25⁺ T cells suppressed the increases in eosinophil numbers and IL-13 levels in BALF seen in SCID mice reconstituted with CD25⁻ spleen cells alone. These results, which are representative of two independent experiments, are expressed as the means $\pm$ SEM between animals ($n = 5$); **, $p < 0.01$.

Induction and analysis of AHR

BALB/c mice were sensitized by three i.p. injections with 10 μ g of OVA and 1.125 mg of aluminum hydroxide (Imject alum; Pierce) in 0.2 ml of saline at weekly intervals. In selected experiments, to deplete CD25⁺ cells BALB/c mice were injected i.v. with either anti-CD25 mAb (PC61; 250 μ g) or control rat IgG four days before sensitization. When indicated, the immune systems of C.B-17 SCID mice were reconstituted with total spleen cells (1×10^6) or CD25⁻ spleen cells (1×10^6) in the presence or absence of purified CD4⁺CD25⁺ T cells (2×10^5) isolated from BALB/c mice 1 day before OVA sensitization. Beginning 1 wk after the last sensitization, mice were challenged daily with 10 μ g of OVA in 25 μ l of saline intra-

nasally (i.n.) for 3–4 days. Where indicated, OVA-specific Th2-polarized cells (1×10^6) were transferred i.v. into naive mice in place of OVA sensitization, followed by i.n. challenge with OVA. Twenty-four hours after the last challenge, we performed bronchoalveolar lavage of lethally anesthetized mice as described previously (19, 20). Bronchoalveolar lavage fluid (BALF) supernatant was stored at -30°C until assayed for cytokines by ELISA. Cells contained in the BALF were subjected to differential cell counts after May-Giemsa stain or FACS analysis after intracellular and/or cell surface staining. Serum samples were obtained by cardiac puncture and centrifugation and then stored at -30°C until being assayed for OVA-specific Ig subclasses by ELISA. Airway responsiveness was measured 24 h after final OVA challenge by recording respiratory pressure curves via whole-body plethysmography (Buxco Electronics) before and after treatment with inhaled methacholine (Sigma-Aldrich) at 10–20 mg/dl for 5 min. Airway reactivity was expressed in enhanced pause (Penh). Histopathology was determined from sections of fixed lungs stained with H&E.

Stimulation of BLN cells

BLN cells obtained from mice 24 h after the last challenge were cultured at 2×10^6 cells/ml with 250 μ g/ml OVA or 1 μ g/ml OVA_{323–339}. Culture supernatants collected after 72 h of culture were assayed for cytokines by ELISA.

Cytokine and Ig ELISA

IL-4, IL-5, IL-10, and IFN- γ levels in culture supernatants or BALF were assayed by ELISA as described (19, 21). The lower detection limits of these assays were 6 pg/ml (IL-4), 50 pg/ml (IL-5), 100 pg/ml (IFN- γ), and 24 pg/ml (IL-10). OVA-specific IgG1 and IgG2a concentrations in serum were assayed using OVA as the capture Ag and biotinylated anti-mouse IgG1 or IgG2a mAb (Caltag Laboratories) as detection Abs. The amount of OVA-specific IgE was determined using a commercial kit (mouse OVA-IgE ELISA kit; Shibayagi) according to the manufacturer's instructions.

Flow cytometry

Cells were stained with biotinylated mAbs specific for CD4 (RM4-5; BD Pharmingen), CCR4 (2G12; to be described in detail elsewhere; Dr. Nagakubo, manuscript in preparation) and CCR7 (EBI-1; eBioscience). Allophycocyanin-conjugated streptavidin (BD Pharmingen) was used as secondary reagent for detection. FITC-, PE-, PE-Cy5-, PerCP-Cy5.5-, or

FIGURE 3. OVA-specific CD4⁺CD25⁺ Treg cells inhibit the development of allergen-induced airway inflammation. The indicated numbers of purified CD4⁺CD25⁺ T cells isolated from DO11.10 mice were transferred into BALB/c mice ($n = 5$ –6), followed by sensitization and challenge with OVA. Twenty-four hours after the final challenge, BALF, BLN, and serum samples were collected. **A**, The transfer of OVA-specific Treg cells reduced the numbers of eosinophils and IL-13 levels in BALF. **B**, The transfer of OVA-specific Treg cells also suppressed Th2-type cytokine production by BLN cells following restimulation in vitro with OVA. **C**, The transfer of OVA-specific Treg cells decreased the serum levels of OVA-specific IgE. These results, which are representative of two independent experiments, are expressed as the means $\pm$ SEM between animals ($n = 5$ –6); *, $p < 0.05$; **, $p < 0.001$; ***, $p < 0.0001$.

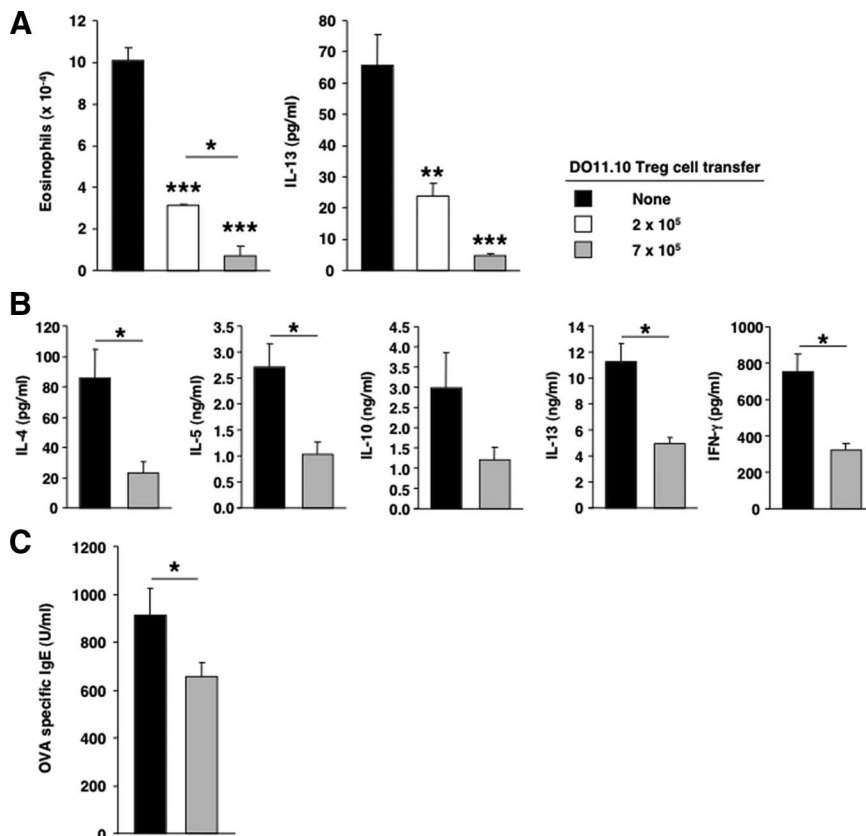
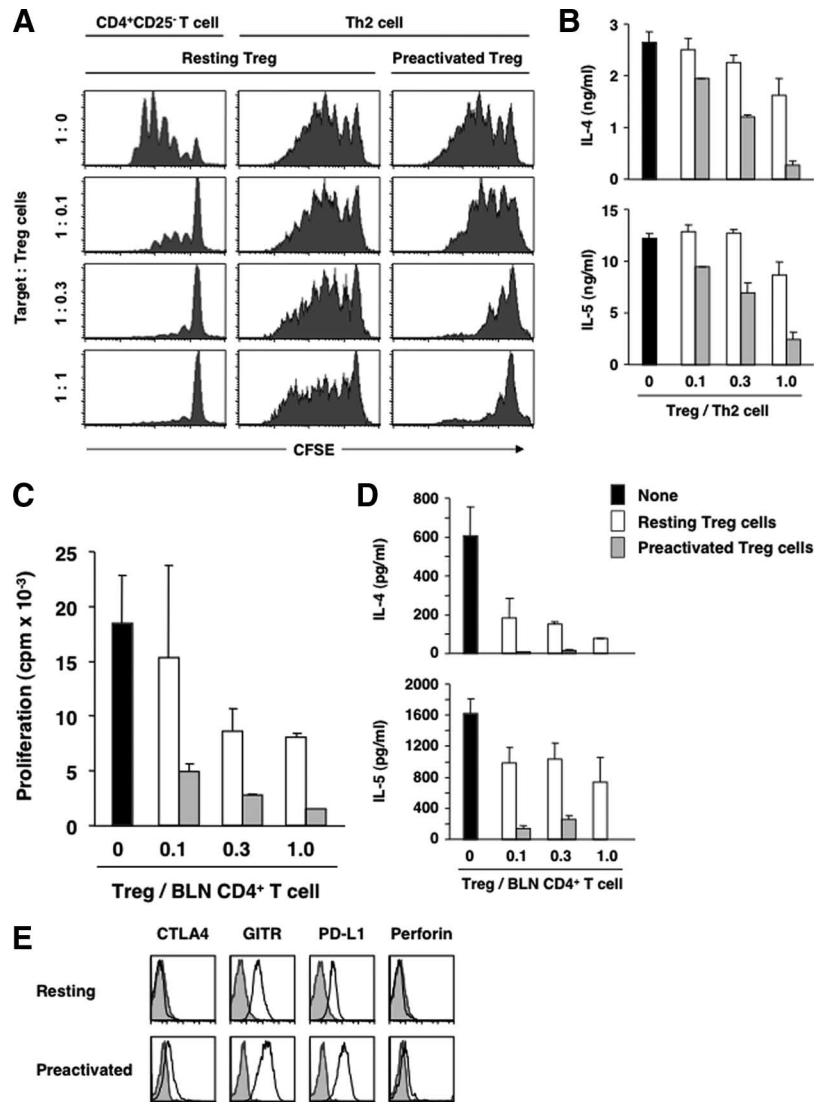


FIGURE 4. Preactivated, but not freshly isolated, CD4⁺CD25⁺ Treg cells efficiently suppress Th2-polarized cells in vitro. CFSE-labeled CD4⁺CD25⁺ T cells, in vitro Th2-polarized cells isolated from DO11.10 mice (A and B), or CD4⁺ T cells derived from the BLN of OVA-sensitized and -challenged BALB/c mice (C and D) were stimulated in the presence of freshly isolated (resting Treg) or anti-CD3/anti-CD28 preactivated (preactivated Treg) CD4⁺CD25⁺ T cells from DO11.10 mice. A and B, Preactivated Treg cells exhibited an increased capacity to suppress the proliferation of (A) and cytokine production by (B) Th2 cells. One representative CFSE-dilution profile displaying CD4⁺KJ1-26⁺ cells is shown in A. C and D, Preactivated Treg cells demonstrated increased ability to suppress proliferation of (C) and cytokine production by (D) CD4⁺ T cells from OVA-sensitized and -challenged mice. E, Preactivated Treg cells expressed higher levels of various regulatory molecules than resting Treg cells. Staining with isotype control Ab is indicated by the gray histogram. These results are representative of three independent experiments. In B–D, the results are expressed as the means $\pm$ SEM of triplicate cultures.



allophycocyanin-conjugated anti-CD4 (RM4-5; BD Pharmingen), anti-DO11.10 TCR (KJ1-26; Caltag Laboratories), anti-CD62L (MEL-14; BD Pharmingen), anti-perforin (eBioOMAK-D; eBioscience), anti-CTLA-4 (UC10-4B9; eBioscience), anti-programmed death ligand 1 (PD-L1; M1H5; eBioscience), anti-glucocorticoid-induced TNFR-related protein (GITR; DTA-1; eBioscience), and anti-CD103 (2E7; eBioscience) Abs, along with the appropriate isotype controls, were also used for staining. Intracellular Foxp3 staining was performed using a commercial Foxp3 staining kit (FJK-16s; eBioscience) according to the manufacturer's instruction. Stained cells were analyzed with FACSscan or FACSCanto (BD Biosciences) cytometer using CellQuest (BD Biosciences) or Flow Jo software (Tree Star), respectively.

Chemotaxis assay

To assess chemotaxis, we used an optically accessible, horizontal chemotaxis apparatus called TAXIScan (Effector Cell Institute). This device can trace the migration of each cell in the channel at time-lapse intervals using a charge-coupled device camera (22, 23). Before assembly of the holder, the surface of the glass plate was coated with RPMI 1640-HEPES containing 10% FCS. Depending on the cell size, etched silicon tips of 3- or 6- μ m depths were used for resting and preactivated Treg cells, respectively. When assembled from an etched silicon tip and a flat glass plate, the holder forms two compartments, one containing cells and the other containing the chemoattractant. Before experimentation, the holder, maintained at 37°C, was filled with RPMI 1640-HEPES medium containing 0.1% BSA. Resting or preactivated Treg cells (1 μ l of 1×10^6 cells/ml) were transferred to one of the two compartments of the holder before 1 μ l of CCL17/TARC or CCL22/MDC (both from R&D systems) solution (where TARC is thymus- and

activation-regulated cytokine and MDC is macrophage-derived cytokine) was injected into the other compartment. Injection marked the beginning of the experiment, followed by the recording of time-lapse images taken every minute for 25 min. Cells in the images were tracked by the program as described previously (22). We calculated velocity and directionality from the migratory pathway data obtained. The direction of cell migration was determined as the angle (radian) toward the concentration gradient; for example, a value of $\pi/2$ would indicate that the cell is migrating toward the concentration gradient, while $-\pi/2$ would indicate that the cell is migrating against the gradient. To compare the chemotactic abilities of resting and preactivated Treg cells, we generated a mean velocity-directionality plot, in which the mean velocity and median directionality of all the cells in a channel were calculated and plotted.

Statistical analysis

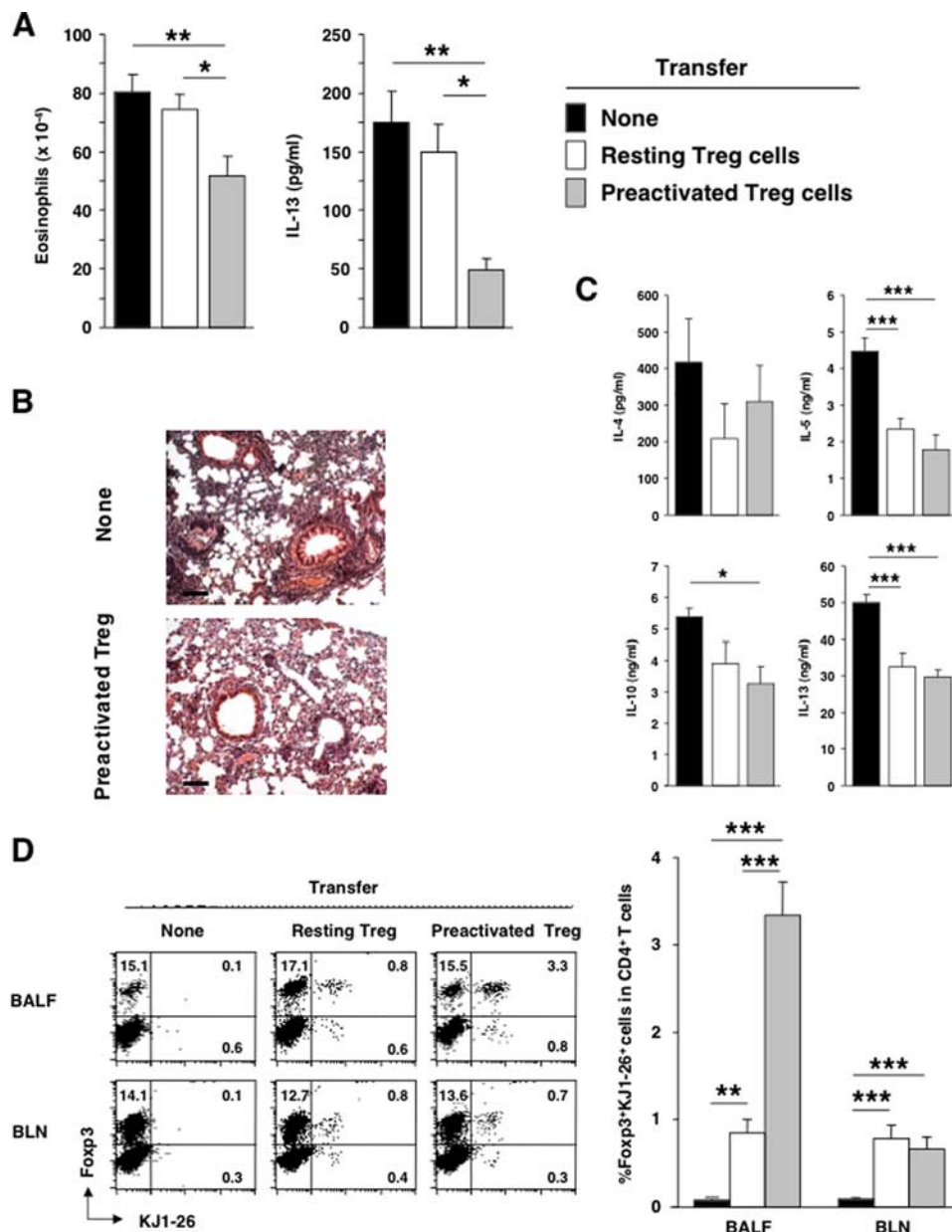
Statistical analyses used the two-tailed Student's *t* test or Fisher's protected least squares difference test on StatView-J 5.0 statistical software (SAS Institute). Results with values of *p* < 0.05 were considered to be statistically significant.

Results

Enhancement of allergic inflammation by depletion of CD4⁺CD25⁺ regulatory T cells

To test the potential suppression of airway inflammation by endogenous CD4⁺CD25⁺ Treg cells, we generated CD25⁺ T cell-depleted BALB/c mice by injection of the anti-CD25 mAb PC61. Examination

FIGURE 5. Transfer of preactivated Treg cells, but not freshly isolated resting Treg cells, influenced airway inflammation in allergen-sensitized mice. Five naive BALB/c mice were sensitized with OVA in alum on days 0, 7, and 14, followed by i.n. challenge with OVA on days 21–24. On day 22, resting or preactivated Treg cells (5×10^5 cells) from DO11.10 mice were adoptively transferred into sensitized mice. Twenty-four hours after the final challenge, BALF, lung, and BLN samples were collected. **A**, Transfer of preactivated Treg cells reduced the numbers of eosinophils and IL-13 levels in the BALF of OVA-sensitized mice. **B**, H&E staining revealed a decrease in the numbers of eosinophils infiltrating into the lungs of mice following the transfer of preactivated Treg cells. Sections are shown that are representative of each group. Bars, 100 μm . **C**, Transfer of preactivated Treg cells suppressed the production of IL-5 and IL-13 by BLN cells restimulated in vitro with OVA. These results are representative of two independent experiments. In **A** and **B**, the results are expressed as the means $\pm$ SEM between animals ($n = 5$). **D**, Preactivated Treg cells efficiently migrated into the lung. The *left* set of figures display representative dot plots detailing Foxp3 and KJ1-26 expression by the CD4^+ population. Aggregate data (two experiments) indicating the proportion of $\text{Foxp3}^+\text{KJ1.26}^+\text{CD4}^+$ T cells within the total CD4^+ T cell population are presented as the means $\pm$ SEM between animals at the right ($n = 3$ –6/group/experiment). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.



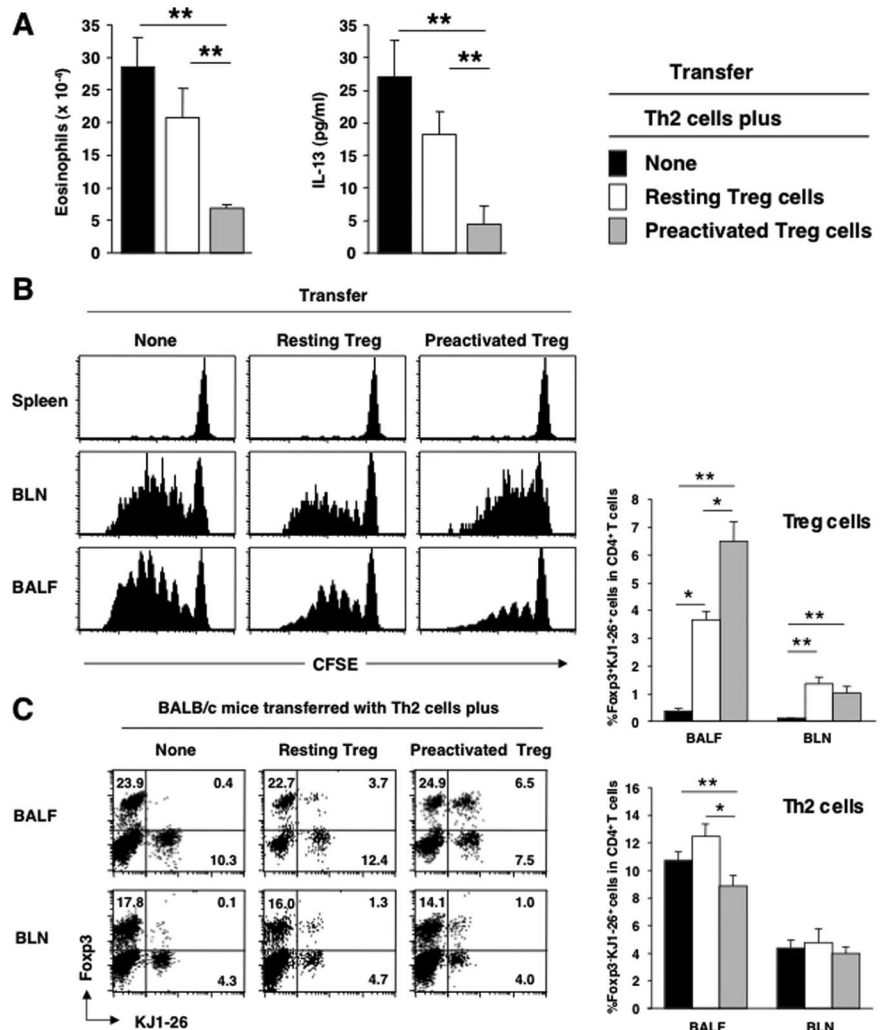
of the CD4^+ population in such mice revealed that CD25^+ T cells, including Foxp3^+ T cells, were effectively eliminated (data not shown). After sensitization and subsequent challenge with OVA, Ab-treated mice demonstrated dramatic increases in both eosinophil numbers and IL-13 levels in BALF in comparison to control IgG-treated mice (Fig. 1A). Treg cell ablation also enhanced eosinophil infiltration into the lung parenchyma (Fig. 1B) and AHR induced by methacholine at a dose of 20 mg/dl in Ab-treated mice ($p < 0.001$; Fig. 1C). To determine the type of airway inflammation occurring in Ab-treated mice, we next examined cytokine production by BLN cells and serum levels of OVA-specific Ig subclasses. Upon in vitro stimulation with OVA, BLN cells prepared from Ab-treated mice after OVA sensitization and challenge produced higher levels of the Th2 cytokines IL-4, IL-5, and IL-13 than those isolated from control IgG-treated, OVA-sensitized/challenged mice (Fig. 1D); the levels of IL-10 and IFN- γ , a Th1-type cytokine, were similar between the two groups of mice (Fig. 1D). Serum levels of OVA-specific IgG1 and IgE, Th2-type Ab isoforms, were significantly increased in Ab-treated mice in comparison to control IgG-treated mice, whereas the levels of the Th1-type isoform IgG2a remained similar (Fig. 1E). We obtained

similar results using C57BL/6 mice (data not shown), clearly indicating that depletion of CD25^+ cells exacerbated the Th2-type airway inflammation seen in mice following sensitization and challenge with OVA Ag.

Suppression of airway inflammation by $\text{CD4}^+\text{CD25}^+$ T cells

Treg cells as well as activated effector T cells, B cells, and dendritic cells can express CD25 on their cell surface (24). Of these cell types, dendritic cells may be involved in suppressing Th2 immunity by facilitating Th1-type immune responses (25). To examine this possibility and confirm the role of $\text{CD4}^+\text{CD25}^+$ T cells in the suppression of Th2-type airway inflammation, we performed reconstitution experiments with SCID mice. Upon sensitization and challenge with OVA, SCID mice reconstituted with CD25^+ cell-depleted spleen cells exhibited dramatic increases in the numbers of eosinophils and IL-13 levels in BALF in comparison to those animals reconstituted with whole spleen cells (Fig. 2). The cotransfer of purified $\text{CD4}^+\text{CD25}^+$ T cells with CD25^+ cell-depleted spleen cells resulted in similar numbers of eosinophils and IL-13 levels in

FIGURE 6. Preactivated Treg cells efficiently migrated into the lung to suppress the influx and activation of Th2 cells in vivo. CFSE-labeled (B) or unlabeled (A and C) Th2-polarized cells (1×10^6) derived from DO11.10 mice were adoptively transferred into naive BALB/c mice with or without resting (5×10^5) or preactivated (5×10^5) Treg cells from DO11.10 mice. Animals were then challenged with OVA for three consecutive days. Twenty-four hours after the last challenge, BALF, BLN, and spleen samples were collected. A, Cotransfer of preactivated Treg cells reduced the numbers of eosinophils and IL-13 levels in the BALF of mice following transfer of OVA-specific Th2 cells. These results, which are representative of three independent experiments ($n = 5$), are expressed as the means $\pm$ SEM. B, Preactivated Treg cells efficiently suppressed the proliferation of Th2 cells in vivo. A representative CFSE-dilution profile displaying $CD4^+KJ1-26^+$ cells, which is representative of three independent experiments, is shown. C, Preactivated Treg cells suppressed the migration of Th2 cells into the lungs of OVA-challenged mice. The left set of figures displays representative dot plots of Foxp3 and KJ1-26 expression for the $CD4^+$ gated population. Aggregate data (three experiments) indicating the proportion of Foxp3 $^+$ KJ1.26 $^+$ CD4 $^+$ and Foxp3 $^-$ KJ1-26 $^+$ CD4 $^+$ T cells within the $CD4^+$ T cell population are expressed as the means $\pm$ SEM between animals and displayed at the upper right and lower right, respectively ($n = 3-4$ /group/experiment); *, $p < 0.05$; and **, $p < 0.01$.



BALF as those seen in mice reconstituted with whole spleen cells (Fig. 2). These results clearly indicated that $CD4^+CD25^+$ T cells, but not other $CD25^+$ T cells, were highly efficient at suppressing Th2-type airway inflammation.

Suppression of airway inflammation by Ag-specific $CD4^+CD25^+$ T cells

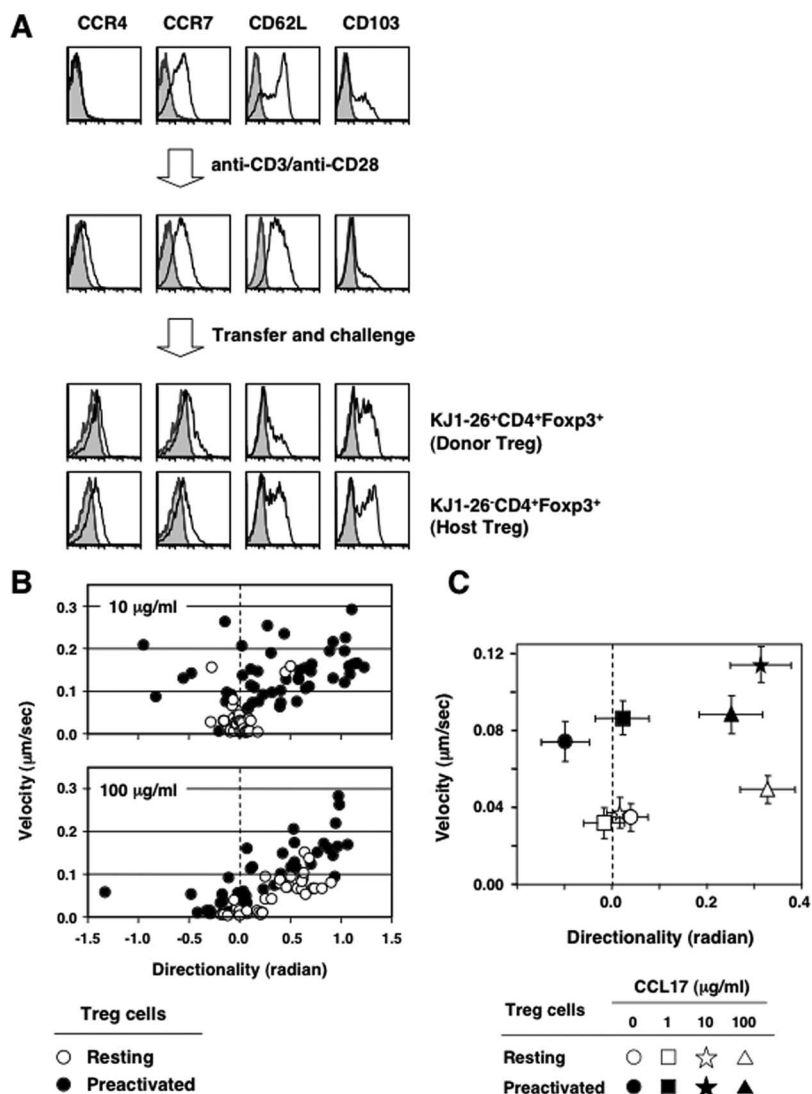
To address the role of Ag-specific $CD4^+CD25^+$ T cells in the suppression of Th2-type airway inflammation, we transferred OVA-specific $CD4^+CD25^+$ T cells isolated from DO11.10 mice into naive BALB/c mice. Transferred Ag-specific $CD4^+CD25^+$ T cells efficiently suppressed the increases in eosinophil numbers and IL-13 levels in BALF seen upon OVA sensitization and airway challenge (Fig. 3A). We isolated BLN cells from mice following transfer of OVA-specific $CD4^+CD25^+$ T cells and then subsequent OVA sensitization and challenge; these cells exhibited markedly reduced production of the Th2-type cytokines IL-4 and IL-5 as well as IL-10 and IFN- γ (Fig. 3B). Following sensitization and challenge with OVA, serum levels of OVA-specific IgE were also significantly reduced in mice that had received transferred OVA-specific $CD4^+CD25^+$ T cells (Fig. 3C). The suppressive effect seen after transfer of Ag-specific $CD4^+CD25^+$ T cells was not observed after the transfer of similar numbers of $CD4^+CD25^+$ T cells isolated from BALB/c mice (data not shown).

Requirement for preactivated Ag-specific $CD4^+CD25^+$ T cells in the suppression of airway inflammation induced in Ag-sensitized mice

Consistent with results reported previously (26), freshly isolated OVA-specific $CD4^+CD25^+$ T cells (resting Treg cells) were inefficient in suppressing proliferation of OVA-specific Th2 cells. In contrast, OVA-specific $CD4^+CD25^+$ T cells that had been preactivated with immobilized anti-CD3/anti-CD28 Abs for 48 h (preactivated Treg cells) strongly inhibited the proliferation of polarized OVA-specific Th2 cells. These preactivated Treg cells suppressed cytokine production by activated OVA-specific Th2 cells. Resting Treg cells were effective only at high E:T ratios (Fig. 4B). Preactivated Treg cells demonstrated efficient suppression of in vitro proliferation of and cytokine production by OVA-stimulated $CD4^+$ T cells isolated from the BLN of OVA-sensitized and challenged mice (Fig. 4, C and D). As shown in Fig. 4E, the increased efficacy of preactivated Treg cells in suppressing target T cell activation was associated with enhanced expression of CTLA-4, GITR, PD-L1, and perforin that have been suggested to constitute the regulatory mechanism of Treg cells (27–30).

We next examined the effect of OVA-specific preactivated Treg cell adoptive transfer on the airway inflammation induced in OVA-sensitized mice. OVA-specific preactivated Treg suppressed airway inflammation more efficiently than OVA-specific resting Treg cells in OVA-presensitized mice following challenge with OVA,

FIGURE 7. Preactivated Treg cells that migrate into the lung express CCR4 and migrate toward TARC in vitro. **A**, We characterized the phenotype of resting Treg cells, preactivated Treg cells, and preactivated Treg cells after transfer. Three BALB/c mice received transfers of Th2 cells (1×10^6 cells) with preactivated (5×10^5 cells) Treg cells from DO11.10 mice. Animals were then treated with an i.n. challenge with OVA for three consecutive days. Twenty-four hours after the last challenge, BALF samples were collected and pooled. Staining with isotype control Ab is indicated by the gray histogram. Histograms representative of three independent experiments are shown. **B** and **C**, Preactivated Treg cells exhibited a greater chemotactic response to CCL17 than resting Treg cells. Individual resting (open symbols) and preactivated (closed symbols) Treg cells were assayed for chemotaxis to CCL17/TARC using a TAXIScan apparatus. **B**, Velocity-directionality plots describing each cell's migration ($n = 50$) are shown. **C**, The mean velocity-directionality plot displays the chemotactic abilities of resting (open symbols) and preactivated (closed symbols) Treg cells in response to 100 (triangle), 10 (star), 1 (square), and 0 $\mu\text{g/ml}$ (circle) CCL17/TARC. Data are expressed as the mean of 50 cells $\pm$ SEM. These results are representative of two independent experiments.



as assessed by eosinophil numbers and IL-13 levels in BALF (Fig. 5A). Eosinophil infiltration in the lung parenchyma was efficiently suppressed by OVA-specific preactivated Treg cells (Fig. 5B). Similar levels of suppression, however, of cytokine production by BLN cells isolated from OVA-prensensitized and -challenged mice were observed after the transfer of OVA-specific resting and preactivated Treg cells stimulated in vitro with OVA (Fig. 5C). Flow cytometric analysis of cells in the BALF and BLN from these mice revealed greater accumulation of the preactivated Treg cells in the lung than that seen for resting Treg cells, whereas both preactivated and resting Treg cells migrated to BLN with a similar efficiency (Fig. 5D).

Efficient suppression of Th2 cell proliferation and migration into the lung by preactivated CD4⁺CD25⁺ T cells

To examine the mechanism by which preactivated OVA-specific CD4⁺CD25⁺ T cells suppressed effector T cell function, we transferred CFSE-labeled OVA-specific Th2 cells into naive BALB/c mice with resting or preactivated Treg cells. After challenge with OVA, the presence of preactivated Treg cells reduced the number of eosinophils and IL-13 level in BALF to a greater degree than that of resting Treg cells (Fig. 6A). This suppression was associated with the poor proliferative responses of the transferred Th2 cells detected in BALF and BLN (Fig. 6B). We also cotransferred

OVA-specific Th2 cells with resting or preactivated Treg cells to determine the effect on the migration of Th2 cells into the lung and BLN. In contrast to animals transferred with only Th2 cells, the accumulation of Th2 cells in the BALF was significantly reduced in mice cotransferred with preactivated, but not resting, Treg cells (Fig. 6C). No differences, however, were seen in the accumulation of Th2 cells in BLN. Consistent with results obtained from sensitized/challenged mice, preactivated Treg cells accumulated at higher levels in BALF than those seen for resting Treg cells, whereas no differences were observed for migration to BLN.

Preactivated CD4⁺CD25⁺ T cells express CCR4 and migrate toward CCR4 ligands

When activated in vitro with immobilized anti-CD3 and anti-CD28 Abs, OVA-specific CD4⁺CD25⁺ T cells isolated from DO11.10 mice up-regulated CCR4 expression and down-regulated CCR7 minimally and CD62L considerably (Fig. 7A). The population of OVA-specific CD4⁺CD25⁺ T cells contained a subset of CD103⁺ cells, the proportion of which was reduced upon stimulation. When we transferred polarized Th2 cells from DO11.10 mice into BALB/c mice with preactivated OVA-specific CD4⁺CD25⁺ T cells and then challenged the animals with OVA, the levels of CCR7 and CD62L on the donor CD4⁺Foxp3⁺ T cells in BALF were further down-regulated. In contrast, CD103 expression on

donor CD4⁺Foxp3⁺ T cells was markedly up-regulated, whereas that of CCR4 remained unchanged.

To demonstrate the biological significance of CCR4 expression by activated OVA-specific Treg cells, we compared the velocity and directionality of resting and preactivated CD4⁺CD25⁺ T cells toward a concentration gradient of the CCR4 ligand CCL17 using a newly developed chemotaxis device, the TAXIScan (22, 23). Preactivated CD4⁺CD25⁺ T cells exhibited an enhanced velocity and directionality in comparison to resting CD4⁺CD25⁺ T cells at every concentration of CCL17 tested (Fig. 7B and supplemental movies).⁴ Overall, preactivated CD4⁺CD25⁺ T cells exhibited enhanced chemotaxis over resting CD4⁺CD25⁺ T cells toward a CCR4 ligand (Fig. 7C), although resting CD4⁺CD25⁺ T cells were capable of significant chemotactic responses at high CCL17 concentrations. Similar results were obtained using another CCR4 ligand, CCL22, as the chemoattractant (data not shown). These results supported the conclusion that preactivated Treg cells were more chemotactically active toward CCR4 ligands than resting Treg cells.

Discussion

In this study, we used reconstitution experiments to demonstrate that allergen-specific preactivated CD4⁺CD25⁺ Treg cells efficiently suppressed airway inflammation in allergen-sensitized mice by accumulating within the lung. Because we sensitized mice with three pulses of OVA to develop strong Th2 responses that would mimic those seen in asthmatic patients, the resulting Th2 cells may be relatively resistant to CD4⁺CD25⁺ Treg cell-mediated suppression. This could explain the decreased effect of resting Treg cells on the suppression of airway inflammation observed in this study in comparison to the previous reports in which only a single allergen sensitization of mice was performed (15). Regardless, our results clearly demonstrated that CD4⁺CD25⁺ Treg cells exert significant suppression of Th2-type airway inflammation in mice. The requirement for preactivation of these Treg cells may have practical relevance for the therapeutic application of Treg cells to the treatment of asthmatic patients who are highly sensitized against causative allergens.

Previously, Stassen et al. established that cytokine production by and proliferation of cultured Th2 cells could only be inhibited by preactivated CD4⁺CD25⁺ T cells, although freshly isolated CD4⁺CD25⁺ T cells could inhibit the IL-4-induced development of Th2 cells from naive CD4⁺ T cells (26). In this study, we demonstrated in vitro that OVA-specific CD4⁺CD25⁺ T cells preactivated with anti-CD3/anti-CD28 (preactivated Treg cells) suppressed the activation of OVA-specific Th2 cells and CD4⁺ T cells from OVA-sensitized and -challenged mice to a greater extent than freshly isolated OVA-specific CD4⁺CD25⁺ T cells (resting Treg cells). This increased efficacy of preactivated Treg cells in suppressing effector T cells was associated with higher levels of CTLA-4, GITR, PD-L1, and perforin expression. Various molecular and cellular events have been described to explain the mechanism(s) of Treg cell-mediated suppression of target T cell activation, such as induction of IDO by B7 interaction with CTLA-4 and/or GITR ligand and subsequent modulation of tryptophan catabolism (27, 28), signaling through the negative costimulatory molecule PD-1 by PD-L1 (29), and induction of cell death by perforin (30). It has been suggested that Treg cells use a combination of some of these mechanisms to control immune responses depending on the milieu of immune responses and activation and/or functional status of target T cells (31, 32). Although the

precise mechanism(s) by which preactivated Treg cells suppress activation of Th2 cells more efficiently than resting Treg cells remains to be determined, it is possible that increased expressions of these regulatory molecules on preactivated Treg cells at the time of encounter with target T cells might contribute, at least in part, to the efficient suppression of Th2 cells. In mice presensitized with OVA, the OVA-specific preactivated Treg cells accumulated in the lung at higher levels than OVA-specific resting Treg cells. When transferred with OVA-specific Th2 cells, preactivated Treg cells suppressed the proliferation of the cotransferred Th2 cells in vivo to a greater extent than resting Treg cells. In contrast, the preactivation status of the OVA-specific Treg cells had no effect on their accumulation in BLN.

We also determined that preactivated OVA-specific Treg cells up-regulated CCR4 expression, resulting in increased migration toward CCR4 ligands than that seen for resting Treg cells. In contrast, we observed the slight and marked down-regulation of CCR7 and CD62L, respectively, on preactivated Treg cells. After adoptive transfer, preactivated Treg cells accumulated at high levels in the lung, down-regulating CCR7 and CD62L and up-regulating CD103 expression. The results suggest that preactivated Treg cells, which express higher levels of CCR4, are recruited with a greater efficiency to the lung, where the CCR4-specific ligands CCL17 and CCL22 were expressed at high levels (17, 18). Migrated cells were further activated to up-regulate CD103 and lose CCR7 expression, which could contribute to their retention and/or accumulation in lung tissue (33, 34). It was recently reported that the loss of CCR4 severely inhibited the accumulation of CD4⁺CD25⁺ Treg cells in the lung (35). CCR4^{-/-} mice also fail to develop allograft tolerance after the administration of anti-CD154 with donor spleen cells, which is associated with a decreased accumulation of Foxp3⁺ Treg cells within the graft (36).

CCR4 has been reported to be expressed on Th2 cells (37, 38). CCR4 expression may also be responsible for the migration of allergen-specific Th2 cells into the lung (39). Studies with mAbs against CCL17 and CCL22, CCR4 ligands, also demonstrated efficacy at controlling AHR when administered to allergen-sensitized mice (40, 41). Thus, CCR4 and its cognate ligands are potential targets for therapeutic interventions in asthma. A recent study, however, revealed that CCR4^{-/-} mice develop airway inflammation and AHR to a similar extent as wild-type mice (42); CCR4 blockade using anti-CCR4 mAb failed to suppress airway inflammation in a mouse model of allergic asthma (43). In this study, while in vitro propagated Th2-polarized cells did not express detectable levels of CCR4 (data not shown), they migrated into the lung and were capable of inducing airway inflammation upon transfer. We also demonstrated that preactivated Treg cells up-regulated CCR4, efficiently migrated into the lung, and suppressed airway inflammation. Host Treg cells present within the lung also expressed CCR4. Therefore, CCR4-blockade could hamper normal Treg cell function in vivo, reducing the utility of CCR4 blockade as a treatment for asthma. Experiments are currently in progress to examine these possibilities.

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Disclosures

The authors have no financial conflict of interest.

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