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Antigen Specificity Is not Required for Modulation of Lung Allergic Responses by Naturally Occurring Regulatory T Cells[1]

Anthony Joetham, Katsuyuki Takeda, Masakazu Okamoto, Christian Taube, Hiroyuki Matsuda, Azzeddine Dakhama, and Erwin W. Gelfand[2]

Naturally occurring Foxp3$^+$CD4$^+$CD25$^+$ T cells isolated from lungs of naive mice regulate lung allergic airway hyperresponsiveness, inflammation, levels of Th2 cytokines, and mucus production. OVA-specific ($\alpha\beta$TCR$^+$) CD4$^+$CD25$^+$ T cells suppressed ragweed-induced airway hyperresponsiveness and inflammation as did anti-TCR-treated OVA-specific CD4$^+$CD25$^+$ T cells, suggesting that Ag-specificity was not required for expression of regulatory activities. Suppression was associated with increased levels of IL-10 and TGF-β; decreased levels of IL-4, IL-5, and IL-13 in bronchoalveolar lavage fluid; and reduced recruitment and activation of CD8$^+$ T cells in the airways. Following intratracheal administration, OVA-specific CD4$^+$CD25$^+$ T cells were identified in both the airway lumens and lung parenchyma, and in some instances in close proximity to host CD8$^+$ T cells. These results demonstrate that the regulatory activities of naturally occurring Foxp3$^+$CD4$^+$CD25$^+$ T cells on lung allergic responses are Ag-nonspecific and thus, independent of Ag-specific recognition. *The Journal of Immunology,* **2009, 183: 1821–1827.**

The low-affinity IL-2 receptor α-chain, CD25, is constitutively expressed on a subpopulation of CD4$^+$ T cells, and is used for isolating and characterizing a population of naturally occurring CD4$^+$CD25$^+$ regulatory T cells (nTregs)[3] (1). CD25, like other constitutively expressed molecules on these cells, CTLA-4, glucocorticoid-induced TNF receptor, TLR4, and CCR5, however, is not unique and in fact can be induced on other cells upon activation. Nevertheless, nTregs differ from the other major classes of T regulatory cells Th3, T_R, and NK T by their constitutive expression of the wing/forkhead transcription factor Foxp3 and an anergic state (2–4). Although CD4$^+$CD25$^+$ T cells in both humans and mice comprise a small fraction (5–10%) of CD4$^+$ T cells and are produced in the thymus, nTregs have been shown to play prominent roles in dictating the outcome of infection, autoimmunity, transplantation, cancer, and increasingly allergy (1). They modulate in vitro and in vivo immune responses through a number of mechanisms, attenuating allergen-driven T cell activation and regulating Th2 immune responses in humans and animals (5–8).

Allergic asthma is an inflammatory disease of the airways characterized by airway hyperresponsiveness (AHR) and eosinophilic inflammation, and increased levels of Th2 cytokines, goblet cell metaplasia, excessive mucus production, elevated Ag-specific IgE, and structural remodeling in the airways. Therapeutically, different approaches have met with various degrees of success in preventing these changes by manipulating various cell types including CD4 (9, 10) and CD8 (11, 12) T cells, NK cells (13), altering levels of cytokines IL-4 (14), IL-5 (15, 16), and IL-13 (12), and reducing levels of Ag-specific Abs (17, 18). With renewed interest in regulatory T cells, the naturally occurring CD4$^+$CD25$^+$ T cells have been recognized as a potentially effective means for influencing the development and outcome of atopic allergic diseases in animals and humans (5–8).

Previously, we demonstrated the ability of CD4$^+$CD25$^+$ nTregs to modulate lung allergic responses through the production and release of the immunoregulatory cytokines IL-10 and TGF-β (19). A novel mechanism of activation and induction of nTreg function was identified and shown to be critically dependent on the engagement/interaction between MHC class I (MHC I) on CD4$^+$CD25$^+$ T cells and CD8 in the lung (20, 21). Both in vitro and in vivo, any interference with this interaction either by blocking or eliminating expression of MHC I on nTregs or blocking expression/accessibility of CD8 resulted in the loss of suppressive activities. These results, however, did not exclude the role of $\alpha\beta$TCRs or address the issue of Ag-specificity in suppression as reported in previous studies (22–26).

There are studies providing evidence supporting intrathymic development and selection as well as the peripheral expansion of nTregs involving self MHC II/peptide diversities (27, 28). Expression of endogenous TCRα-chains may be necessary in some situations for the development of nTregs (29–31) but not in TCR transgenic RAG$^{-/-}$ mice expressing the OVA peptide (32) or RAG$^{-/-}$ mice expressing influenza hemagglutinin under the control of the Ig k promoter (33). Thus, the requirement for Ag-specific TCR repertoire modification and selection (34) and specific Ag/TCR engagement for activation and expression of suppressive activities in vivo is not entirely defined. The findings that adoptive transfer of Ag-specific Foxp3-transduced T cells but not polyclonal regulatory T cells prevented diabetes (24) appeared at odds with the protection against development of spontaneous autoimmune encephalomyelitis conferred on myelin basic protein-specific transgenic recipients that received normal wild-type (WT)

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[3] Abbreviations used in this paper: nTreg, naturally occurring CD4$^+$CD25$^+$ regulatory T cell; AHR, airway hyperresponsiveness; MHC I, MHC class I; WT, wild type; RW, ragweed; R_L, lung resistance; BAL, bronchoalveolar lavage.

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$CD4^+$ T cells (25). Similarly, Ag-specific and nonspecific nTregs were equally effective in preventing delayed type hypersensitivity (26) and once activated via the TCRs in vitro, the suppressive function of $CD4^+CD25^+$ from transgenic donors was shown to be Ag-nonspecific (22).

In the present study, we investigated the requirement for Ag-specificity and involvement of the TCR in the induction and expression of suppressive activities of nTregs. $CD4^+CD25^+$ T cells expressing an OVA-specific $\alpha\beta$TCR from naive DO11.10 donors or treated with an Id-specific Ab (KJ1.26), which prevents the binding of OVA to the OVA-specific $\alpha\beta$TCRs (35), were shown to be effective in suppressing unrelated (ragweed, RW) allergen-induced lung allergic immune responses, similar to the effects of nTregs with no known Ag-specific TCR (19–21). Together, the data support the Ag-independent modulation of lung allergic responses by naturally occurring T regulatory cells.

Materials and Methods

Animals

Pathogen-free, 6- to 8-wk-old female BALB/c mice were obtained from The Jackson Laboratory, and DO11.10 mice, which express a TCR transgene specific for OVA peptide were provided by Dr. Philippa Marrack (National Jewish Health, Denver, CO). All mice were maintained on an OVA-free diet and all protocols were approved by the Institutional Animal Care and Use Committee of National Jewish Health.

Sensitization

Sensitization was conducted by i.p. injection of either 20 μg RW (Greer Laboratory) or 20 μg OVA, both emulsified in 2.0 mg alum hydroxide (AlumImject; Pierce) in a total volume of 100 μl on days 1 and 14. Sensitized and sham-sensitized littermates received aerosol challenges for 20 min each day on 3 consecutive days (days 26, 27, and 28) with 1% RW or OVA in PBS using an ultrasonic nebulizer (Omron) (denoted as OVA/OVA or RW/RW for sensitized and challenged or PBS/OVA or PBS/RW for sham-sensitized and challenged) (13).

Cell preparation and culture

OVA-specific $CD4^+CD25^+$ (OVA-$CD4^+CD25^+$) and $CD4^+CD25^-$ T cells from naive DO11.10 donors were isolated by collagenase digestion of lungs and enriched using nylon wool columns as described previously (19). Lymphocytes were further purified by $CD4^+CD25^+$ regulatory T cell MACS beads (Miltenyi Biotec), resulting in a purity of >95% $CD4^+CD25^+$ cells, and by sorting on MoFlo (DakoCytomation) following staining with KJ1.26 Ab, providing a purified population of >99% OVA-specific $CD4^+CD25^+$ T cells. The $CD4^+CD25^-$ populations contained <5% $CD25^+$ cells.

Cells were washed, counted, and resuspended to a final concentration of 4×10^6 cells per ml in RPMI 1640 tissue culture medium (Mediatech Celgro), containing heat-inactivated FCS (10%; Sigma-Aldrich), L-glutamine (5 mM), 2-ME (2 mM), HEPES buffer (15 mM), penicillin (100 U/ml), and streptomycin (100 μg/ml) (all from Life Technologies).

Adoptive transfer

Recipient mice received 5×10^5 isolated lung OVA-specific $CD4^+CD25^+$ or anti-TCR (KJ1.26)-treated OVA-specific $CD4^+CD25^+$ T cells (anti-TCR/$CD4^+CD25^+$) intratracheally in 50 μl of PBS before allergen challenge.

In some adoptive transfer experiments, $CD4^+CD25^+$ T cells were treated with anti-MHC I (200 μg/ml, 34–1-2S, eBiosciences) or control rat IgG in vitro for 1 h and washed extensively with PBS before transfer.

Antibodies

mAbs from the culture supernatants of the IgG-producing hybridomas KJ1.26 (anti-OVA TCR, provided by Dr. Philippa Marrack, National Jewish Health, Denver, CO), 53–6.7 (anti-CD8α), and 53–5.8 (anti-CD8β) (American Type Culture Collection, Manassas, VA) were purified by protein G chromatography. Anti-CD8α reacts with the 38 and 34 kDa α-chains of the CD8 Ag (Ly-2 or Lyt-2) of all mouse strains, while anti-CD8β reacts with the β-chain (Ly-3.2 or Lyt-3.2) of most mouse strains (200 μg/ml). Anti-mouse NK (anti-mouse asialo GM1, Cedarlane) (800 μg) was injected i.v. before allergen challenge and anti-mouse CD8 (50 μg) or control rat IgG (50 μg) was administered by microspray intratracheally using a microsprayer (Penn-Century) before and immediately following intratracheal transfer of $CD4^+CD25^+$ T cells (19). There were no differences observed when either anti-CD8α or anti-CD8β was used.

Measurement of airway responsiveness

Airway responsiveness, 48 h following the last challenge, was assessed as a change in airway function to increasing concentrations of aerosolized methacholine administered for 10 s (60 breaths/min, 500 μl tidal volume). Lung resistance (R_L) was continuously computed (Labview, National Instruments) by fitting flow, volume, and pressure to an equation of motion. Maximum values of R_L were taken and expressed as a percentage change from baseline following saline aerosolization. Among all treatment groups, there were no significant differences in baseline (saline) lung resistance values.

Bronchoalveolar lavage (BAL)

Immediately following measurement of AHR, lungs were lavaged (1×1 ml 37°C). Total leukocyte numbers were counted (Coulter Counter, Coulter Corporation). Differential cell counts were performed under light microscopy by counting at least 200 cells on cytocentrifuged preparations (Cytospin 2; Cytospin, Shandon), stained with Leukostat (Fisher Diagnostics) and differentiated by standard hematological procedures.

Determination of serum Ab titers by ELISA

Serum levels of total IgE, RW-specific IgG1, IgG2a, and IgG2b were measured by ELISA. Total IgE levels were calculated by comparison with known mouse IgE standards (BD Pharmingen).

Measurement of cytokine levels

Cytokine levels in the BAL fluid and in supernatants of in vitro cultured lung cells were measured by ELISA (IL-4, IL-5, IL-10, IFN-γ, TGF-β) kits from BD Pharmingen and IL-13 using kits from R&D Systems; ELISAs were performed according to the manufacturers' directions. The limits of detection were 4 pg/ml for IL-4 and IL-5, 10 pg/ml for IL-10 and IFN-γ, 8 pg/ml for IL-13, and 6 pg/ml for TGF-β.

FACS analysis

Enriched lung and BAL cells, following preincubation with naive mouse serum in staining buffer (PBS, 2% FCS, 0.2% sodium azide), were labeled with the following conjugated Abs purchased from BD Pharmingen: anti-CD3 FITC, PE, PerCP, APC (17A2); anti-CD4 FITC, PE, PerCP, APC (L3T4); anti-CD25 FITC (7D4), PE (PC61); anti-CD8α FITC, PE, PerCP (53–6.7); anti-CD122 PE (TM-β1); anti-panNK FITC (DX5), anti-H-$2k^d$ FITC, PE. For intracellular staining, cells were stimulated with PMA (100 ng/ml) and ionomycin (2 μg/ml, Sigma-Aldrich) in complete medium overnight and for 6 h in the presence of brefeldin A (10 μg/ml, Sigma-Aldrich). Cells were fixed with 4% formaldehyde in PBS, permeabilized in 0.5% saponin, and stained with anti-IL-10 PE, APC (JES5–16E3): IFN-γ PE, APC (XMG1.2); Foxp3 PE and TGF-β (A75–3.1) (eBioscience). Fluorochrome (FITC, PE, PerCPAPC)-labeled isotype-matched control Abs were used for background fluorescence staining. Staining was analyzed on a FACScalibur flow cytometry (BD Pharmingen) using CellQuest Pro software (BD Biosciences). Fluorescence intensity was compared with cells stained with corresponding labeled isotype-matched controls.

Immunofluorescent histochemistry

Lungs were fixed by inflation in 1 ml of 40% OCT compound and frozen in OCT (Sakura Finetek) or formalin and immersion in 10% formalin. Frozen sections were air-dried, fixed with acetone, and stained with purified rat anti-mouse CD8 followed by staining with Cy3-goat anti-rat IgG diluted in TBS/1% BSA/0.05% Tween 20 with several washings with TBS in between steps. Naive rat serum and Fc-block (4G2) were used to limit nonspecific binding before incubation with biotinylated KJ1.26 and streptavidin-FITC. Cover glasses were affixed with mounting medium containing 4′,6-diamidino-2-phenylindole (Vector Laboratories). Slides were analyzed using an epifluorescence microscope (Nikon Eclipse 2000) equipped with a Cool-Snap CCD camera and Metamorph image analysis software (Molecular Devices). Exposure times were chosen in a manner that limited background (no cells or OVA-TCR^- cells) staining and only signal above background was analyzed. Morphometric data were determined by blinded examination of four to six fields per slide of four samples per group.

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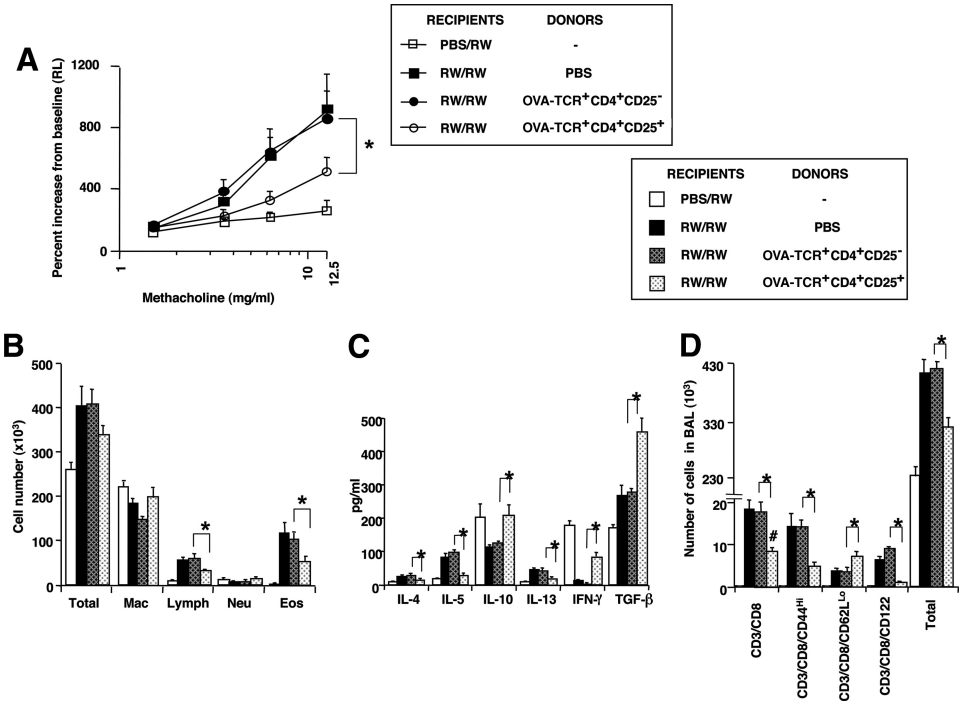

FIGURE 1. Effect of transfer of nTregs from naive DO11.10 donors into RW sensitized and challenged BALB/c recipients. Sensitized BALB/c mice received nTregs before challenge. *A*, AHR; *B*, BAL fluid inflammatory cell composition; *C*, BAL cytokine levels; and *D*, Activation status of isolated lung $CD3^+CD8^+$ T cells. Shown are the means ± SEM from three independent experiments (four mice/group, $n = 12$ mice analyzed for each group). *, $p < 0.05$ comparing the suppressive activity of nTregs in sensitized and challenged recipients to recipients of non-Tregs ($CD4^+CD25^-$ T cells).

Statistical analysis

ANOVA was used to determine statistical significance. Comparisons for all pairs were performed by Tukey-Kramer highest significant difference test. The p values for significance were set to 0.05. Values for all measurements were expressed as the mean ± SEM.

Results

OVA-TCR$^+$CD4$^+$CD25$^+$ T cells suppress RW-induced AHR and inflammation

The aim of these experiments was to investigate the regulatory capacity of nTregs isolated from naive DO11.10 donors, which express a TCR transgene specific for OVA peptide, on airway responsiveness and inflammation induced by sensitization and challenge of BALB/c mice with an unrelated allergen RW. Following sensitization and airway challenge to RW, mice developed increased AHR measured as increases in R_L in response to increasing doses of inhaled methacholine when compared with nonsensitized but challenged mice (Fig. 1*A*). Intratracheal administration of OVA-TCR$^+$CD4$^+$CD25$^+$ but not OVA-TCR$^+$CD4$^+$CD25$^-$ T cells resulted in a significant decrease in AHR, consistent with previous reports where naive nTregs were effective in suppressing AHR (19–21).

Associated with the increases in AHR, the number of airway eosinophils were significantly increased in RW sensitized and challenged mice (Fig. 1*B*). The allergen-induced airway eosinophilia (and increase in lymphocyte numbers) was significantly reduced in mice which received OVA-TCR$^+$CD4$^+$CD25$^+$ but not OVA-TCR$^+$CD4$^+$CD25$^-$ T cells.

Following sensitization and challenge with RW, but not challenge alone, increases in the levels of Th2 cytokines in BAL fluid were detected. A significant increase in levels of IL-4, IL-5, and IL-13, and decreases in IL-10 and IFN-γ were detected in sensitized and challenged mice given PBS or OVA-TCR$^+$CD4$^+$CD25$^-$ T cells but not challenged only animals. BAL TGF-β levels in both of these groups of sensitized and challenged mice also increased compared with mice that were challenged alone. Intratracheal transfer of OVA-TCR$^+$CD4$^+$CD25$^+$ T cells to RW sensitized and challenged recipients resulted in a significant reduction in the levels of IL-4, IL-5, and IL-13, accompanied by increases in the levels of IL-10 and IFN-γ (Fig. 1*C*). A further increase in the levels of TGF-β in BAL was also detected in the recipients of OVA-TCR$^+$CD4$^+$CD25$^+$ but not OVA-TCR$^+$CD4$^+$CD25$^-$ T cells as shown in Fig. 1*C*.

When serum levels of RW-specific Abs were examined, sensitized and challenged mice showed increases in Abs of all isotypes (IgE, IgG1, IgG2, IgG2b) and there was little difference among the three groups (data not shown), consistent with previous reports (19–21). The absence of regulatory effects on specific Ab production, particularly IgE Ab, likely reflects that sensitization with alum was completed before regulatory T cell transfer.

OVA-TCR$^+$CD4$^+$CD25$^+$ T cells decrease the activation of cells in the airways

Previously, naturally occurring $CD4^+CD25^+$ regulatory T cells from naive mice bearing no known allergen-specific TCRs such as those from the DO11.10 mice were shown to regulate CD8-mediated lung allergic responses (20, 21). Following sensitization and challenge, but not challenge alone, a significant increase in the number of lymphocytes was detected in the airways, including numbers of $CD8^+$ T cells (Fig. 1*D*). In sensitized and challenged mice given PBS or $CD4^+CD25^-$ T cells, a significant number of $CD8^+$ T cells expressed the activation markers $CD44^{high}$, $CD122^{high}$, and $CD62L^{low}$. Following transfer of OVA-TCR$^+$CD4$^+$CD25$^+$ regulatory T cells, total cell number and numbers of $CD8^+$ T cells were significantly reduced. In addition, fewer $CD8^+$ T cells expressed the characteristic markers of activation $CD44^{high}$, $CD122^{high}$, and $CD62L^{low}$ in these recipients, indicating that OVA-TCR$^+$CD4$^+$CD25$^+$ regulatory T cells may regulate $CD8^+$ T cell-mediated lung allergic responses by preventing both the activation of $CD8^+$ T cells and their accumulation in the airways.

Taken together, these data demonstrated that nTregs that express a TCR transgene for OVA were fully effective in suppressing all of the allergic airway responses in RW sensitized and challenged mice.

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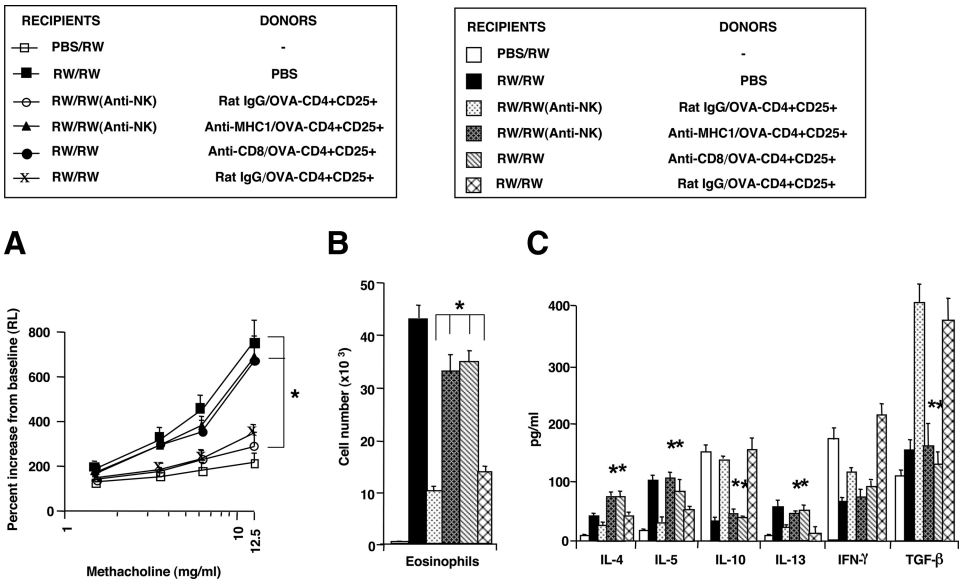

FIGURE 2. Effect of in vitro anti-MHC I treatment of nTregs and in vivo administration of anti-CD8α on lung allergic responses in RW sensitized and challenged recipients. Isolated nTregs from DO11.10 donors were treated with anti-MHC I Ab (or control rat IgG) in vitro for 1 h before transfer into sensitized and challenged recipients depleted of NK cells. In vivo, anti-CD8β (50 μg) (or control rat IgG, 50 μg) was administered intratracheally to sensitized and challenged mice before transfer of nTregs. *A*, AHR; *B*, BAL cell composition; and *C*, BAL cytokine levels. Results represent means ± SEM from three independent experiments, $n = 12$. *, $p < 0.05$ comparing the suppressive activity in NK cell-depleted recipients of untreated nTregs compared with anti-MHC I-treated nTregs or anti-CD8-treated recipients.

Anti-CD8 treatment of recipients or anti-MHC I treatment of nTregs attenuates suppression of allergic AHR and airway inflammation and the increases in IL-10 and TGF-β

Previously, we demonstrated that the interaction of MHC I on nTregs and CD8 in the host lung was essential for the activation of the regulatory functions of nTregs (20). This role for CD8 was recently supported by data suggesting that CD8$^+$ T cells enhanced the regulatory function of CD4$^+$CD25$^+$ T cells in a different animal model (36). We investigated the effects of in vitro treatment of OVA-CD4$^+$CD25$^+$ T cells with anti-MHC I or administration of anti-CD8β by microspray, before intratracheal transfer of OVA-CD4$^+$CD25$^+$ T cells in RW sensitized and challenged mice. To avoid host NK cell elimination of cells lacking expression of MHC I class molecules (37, 38), we first depleted NK cells in recipient mice as previously reported (20). FACS analysis of spleen cells from mice treated with anti-NK Ab (anti-asialo-GM1) showed that nearly all NK cells were depleted; following staining with the pan-NK Ab, < 0.7% of spleen cells stained positively compared with 2.7% in mice receiving control Ab (data not shown). In mice depleted of NK cells, OVA-TCR$^+$CD4$^+$CD25$^+$ T cells (treated with control rat IgG) maintained suppressive activity-reducing AHR (Fig. 2*A*). In contrast, OVA-TCR$^+$CD4$^+$CD25$^+$ T cells treated with anti-MHC I Ab before intratracheal administration in mice depleted of NK cells failed to suppress the development of AHR (Fig. 2*A*), airway eosinophilia (Fig. 2*B*), or increases in Th2 cytokine levels. The increases in levels of IL-10 and TGF-β were prevented by anti-MHC I treatment (Fig. 2*C*).

Similarly, intratracheal treatment of recipients with anti-CD8β Ab but not control rat IgG before transfer of OVA-TCR$^+$CD4$^+$CD25$^+$ T cells led to the elimination of suppressive activities and the sensitized mice remained fully responsive to allergen challenge with development of AHR (Fig. 2*A*), airway eosinophilia (Fig. 2*B*), and a cytokine profile similar to controls including increased levels of Th2 cytokines and reduced levels of IL-10 and TGF-β (Fig. 2*C*).

Together, the data demonstrated that the functional activation and induction of suppressive activities of OVA-TCR$^+$CD4$^+$CD25$^+$ T cells on allergen-induced lung responses was dependent on the interaction between MHC I on nTregs and CD8 in the host lung, and that the engagement of the Ag-specific TCR was not essential for expression of their regulatory function. In these ways, the OVA-nTregs were identical with the nTregs obtained from WT mice.

OVA-TCR$^+$CD4$^+$CD25$^+$ T cells treated with anti-TCR-specific Ab anti-TCR/CD4$^+$CD25$^+$ suppress development of AHR and inflammation

The data above supported the concept that nTregs regulate lung allergic immune responses in an Ag-nonspecific fashion. However, as involvement of the TCR has been invoked by studies showing that TCR stimulation by anti-CD3 or Ag-specific TCR modification augmented suppression (22–25), we confirmed that the Ag-specific TCR was not required for suppression of lung allergic responses. In this study, we determined the effects of blocking the TCR with a specific Ab, KJ1.26, on the regulatory activities of CD4$^+$CD25$^+$ T cells isolated from the DO11.10 mice. This Ab binds the TCR for OVA on T cells and blocks completely the responses of DO11.10 hybridoma cells to OVA/H-2^b and OVA/H-2^d (35). Following isolation, anti-TCR/CD4$^+$CD25$^+$ T cells were further purified (>99%) by staining with a saturated concentration of Ab and sorted on the basis of bound KJ1.26 before intratracheal administration and allergen challenge in concordant (OVA) and discordant (RW) Ag-sensitized and challenged recipients. Despite blocking the TCR, anti-TCR/CD4$^+$CD25$^+$ T cells maintained their regulatory activities and suppressed development of AHR in both OVA- and RW-sensitized and challenged recipients (Fig. 3*A*), similar to untreated OVA-TCR/CD4$^+$CD25$^+$ T cells (Fig. 1*A*). As shown in Fig. 3*B*, OVA sensitization and challenge induced a significant increase and greater numbers of eosinophils in BAL than RW. Following the transfer of anti-TCR/CD4$^+$CD25$^+$ T cells, however, both OVA and RW allergen-induced airway eosinophilia was significantly reduced. Similar to untreated nTregs, anti-TCR-nTregs remained effective in reducing the levels of IL-4, IL-5, and IL-13, and increasing the levels of IL-10 and TGF-β in BAL fluid (Fig. 3*C*).

Collectively, these data demonstrated the functional activation of CD4$^+$CD25$^+$ regulatory T cells independent of the TCR, further demonstrating that Ag-specificity and the TCR were not required for expression of suppressive activities.

Identification of OVA-TCR$^+$CD4$^+$CD25$^+$ T cells in lung tissue

To date, there are few reports on the distribution of naturally occurring CD4$^+$CD25$^+$ regulatory T cells in lung tissue following adoptive transfer. Exploiting the fact that transferred cells expressed an Ag-specific TCR and the availability of an Ab specific to this TCR, we identified the transferred CD4$^+$CD25$^+$ T cells in

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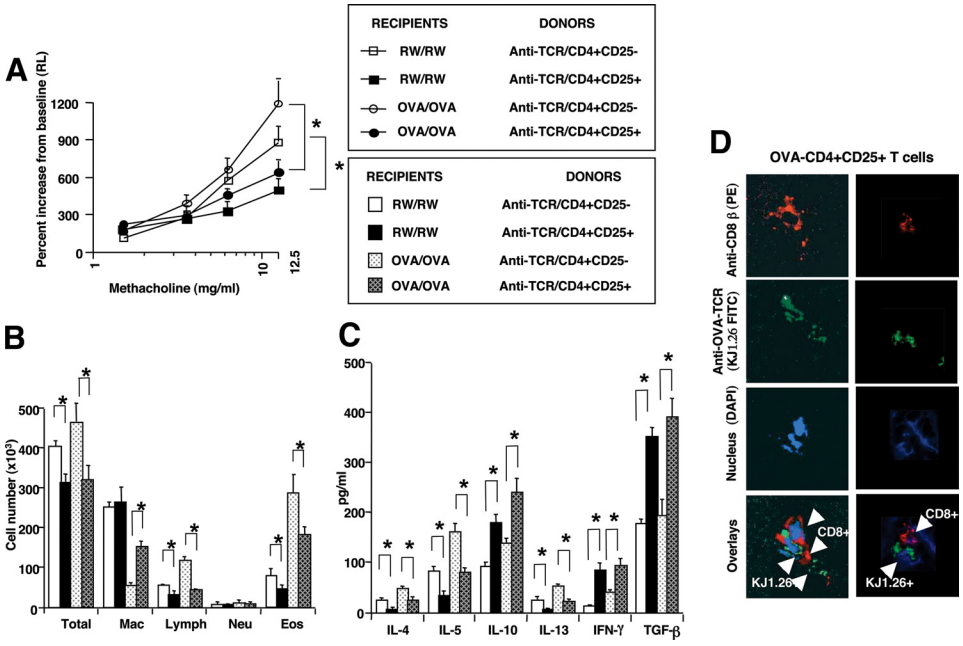

FIGURE 3. Effect of treating OVA-TCR$^+$CD4$^+$CD25$^+$ T cells with anti-KJ1.26 in OVA or RW sensitized and challenged recipients. nTregs were isolated from naive DO11.10 mice and sorted with KJ1.26 Ab before transfer into WT recipients *A*, AHR; *B*, BAL cell composition; *C*, BAL cytokine levels; and *D*, Demonstration of CD8-nTreg contacts (in two examples) in lung parenchymal tissue of RW sensitized and challenged recipients (oil immersion, ×1000). Results are shown as means ± SEM from three independent experiments, $n = 12$. *, $p < 0.05$ comparing the suppressive activity of anti-KJ1.26-treated CD4$^+$CD25$^+$ and CD4$^+$CD25$^-$ T cells in OVA or RW sensitized and challenged recipients.

lung sections. Frozen sections of lung from recipient mice given OVA-CD4$^+$CD25$^+$ regulatory T cells from naive DO11.10 donors were monitored by immunohistochemical staining. Host CD8$^+$ T cells were first identified using rat anti-mouse CD8β and counterstaining with goat anti-rat IgG Cy3 (red). Transferred nTregs were identified using biotinylated KJ1.26 and strepavidin-FITC (green), and cell nuclei with DAPI staining (blue). Fig. 3*D* illustrates the individual cell staining patterns as well as composites or overlays of labeled colors detected. Host CD8$^+$ T cells were detected in both lung parenchyma and the airways lumens in all of the recipients and transferred CD4$^+$CD25$^+$ T cells were identified in close proximity to host CD8$^+$ T cells in recipients of OVA-TCR$^+$CD4$^+$CD25$^+$. Despite an elapse of 96 h following intratracheal administration, transferred cells remained in the lung and could be identified immunohistochemically.

Discussion

nTregs, a small subset of $\alpha\beta$ T cells, express diverse TCRs with a greater bias toward self-Ags. This interaction between TCRs and self-Ags is proposed as important for the development and selection of nTregs in the thymus (27, 28). Unlike conventional CD4$^+$CD25$^-$ T cells, which recognize foreign Ags in the context of MHC II and $\alpha\beta^+$TCRs, the evidence for a requirement or involvement of Ag-specific TCRs on nTregs in the induction and expression of regulatory function in vivo is limited and somewhat conflicting. In this study, using a series of complementary approaches, we demonstrated that Ag-specific TCRs on naturally occurring Foxp3$^+$CD4$^+$CD25$^+$ T cells isolated from naive donors were not essential for the induction and expression of suppressive activities which resulted in the reduction of allergen-induced AHR and inflammation. This activation and expression of regulatory function was dependent on the interaction/engagement of MHC I and CD8, confirming earlier results in naive WT nTregs (20). Both in vitro and in vivo inhibition or interference with the interaction/engagement of MHC I on nTregs and CD8 in the lungs of recipient mice was shown to effectively prevent the expression of regulatory activities.

Following RW sensitization and challenge, BALB/c mice developed significant AHR and eosinophilic inflammation. Concomitantly, BAL levels of the Th2 cytokines IL-4, IL-5, and IL-13 were elevated, and levels of IFN-γ and IL-10 were low. TGF-β levels increased slightly in sensitized and challenged mice compared with those challenged alone. When these sensitized and challenged mice received nTregs from DO11.10 mice expressing a TCR transgene for OVA, all lung allergic responses were reduced, accompanied by increases in IL-10 and TGF-β. IL-10 and TGF-β have been shown to play critical roles in nTreg suppression (8, 19–21).

Under all of the conditions tested, recipients of OVA-CD4$^+$CD25$^-$ T cells exhibited no suppression of lung allergic responses. These results suggested that nTreg suppression was not Ag-specific. Although the TCRs on transferred nTregs expressed a transgene specific for OVA, they fully suppressed RW-induced responses similar to WT nTregs. Theoretically, Ag mimicry toward an unrelated Ag could possibly activate the nTregs through the TCR resulting in suppression of the lung allergic responses to another Ags. To eliminate this possibility, isolated nTregs from DO11.10 mice were further identified by staining with KJ1.26, an Ab which has been shown to specifically and completely block OVA-specific TCRs by Marrack et al. (35), and then sorted based on bound KJ1.26 Ab before transfer to achieve an even greater purification of the cells to >99% KJ1.26$^+$ cells. Interestingly, anti-TCR treatment and sorting of these OVA-specific CD4$^+$CD25$^+$ T cells did not alter their suppressive activities as demonstrated by the significant reduction of lung allergic responses induced by either the relevant Ag OVA or the unrelated allergen RW, similar to the suppressive effects seen with untreated nTregs (19–21). Corresponding to the reduction in AHR and inflammation; lower levels of IL-4, IL-5, and IL-13; and increased levels of IL-10 and TGF-β were detected in the BAL fluid. These data further supported the notion that Ag-specific TCRs on nTregs did not appear essential for the in vivo induction of regulatory activities.

We were also able to take advantage of the ability to identify these TCR (KJ1.26)-expressing cells in lung tissue following transfer. There are no reports on the localization of nTregs following transfer in the lungs of sensitized mice exposed to allergen challenge. The transferred KJ1.26$^+$ nTregs were shown to be distributed throughout the lung tissue, up to 96 h after transfer, and in many cases, were shown to be in close contact to CD8$^+$ T cells

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throughout the lung parenchyma and central airways. We previously established in vitro that the suppressive activity of nTregs, exhibited by increased IL-10 and TGF-β levels following interaction with $CD8^+$ T cells was indeed contact dependent (19).

The important role of $CD8^+$ T cells in the development of lung allergic responses has been demonstrated by their depletion in sensitized and challenged WT mice resulting in lower AHR and inflammation (9), and reconstitution with primed but not naive $CD8^+$ T cells or differentiated $CD8^+$ effector memory T cells in sensitized and challenged $CD8^{-/-}$ recipients (12, 20). CD8 expression has also been implicated in the activation of the suppressive program of nTregs (36). In the absence of any obvious Ag specificity, adoptive transfer of nTregs from naive donors suppressed AHR and inflammation in recipients reconstituted with negatively but not positively selected primed $CD8^+$ T cells (20). In the present study, we extended the investigations of the specific role of host $CD8^+$ T cells. Following sensitization and challenge but not challenge alone, the number of lymphocytes increased in BAL fluid. The increase was due in part to greater numbers of $CD8^+$ T cells accumulating in the airways. When their phenotype was further characterized, they had increased expression of the activation markers CD44, CD122, but low expression of CD62L. Following transfer of OVA-specific nTregs from DO11.10 mice, these cells were effective in suppressing the unrelated RW-induced lung allergic responses by reducing the numbers of total cells and of $CD8^+$ T cells and fewer $CD8^+$ cells expressed the activation markers. It was previously shown that activated $CD8^+$ T cells are an important source of the proinflammatory cytokine IL-13 (12). The observed reductions in the BAL levels of IL-13 following transfer of the $CD4^+CD25^+$ nTregs was likely due, at least in part, to the decrease in number and activation of $CD8^+$ T cells in the lungs of recipient mice. The inhibition of activation and decreased accumulation of $CD8^+$ T cells into the airways can be attributed directly to nTreg activity in the challenge phase since the adoptive transfer of nTregs was always after completion of the sensitization phase. This is supported by the findings that nTreg transfer after sensitization and before challenge had little effect on specific Ab levels, including RW- or OVA-specific IgE Ab. The results of nTreg transfer on lung allergic responses are consistent with findings of other investigators demonstrating that nTregs with and without Ag specificity are effective in preventing in vitro proliferation of both $CD4^+$ and $CD8^+$ T cells and preventing or reducing the development of disease in various animal models (5–8).

Not only are $CD8^+$ T cells important effector cells in the development of lung allergic responses, we previously demonstrated an important role for CD8 together with MHC I expression on Tregs in the activation of nTreg suppressive activity (20). Recent data confirmed the role of $CD8^+$ T cells in activating the suppressive program of $CD4^+CD25^+$ T cells in a different mouse (C57BL/6) model (36). In the present study, we determined whether or not the same mechanism of activation of OVA-$CD4^+CD25^+$ T cells occurs in BALB/c mice sensitized and challenged with the unrelated allergen RW. As NK cells were reported to efficiently remove any cells lacking MHC I on the cell surface (37, 38), sensitized recipient mice were first depleted of NK cells before the intratracheal transfer of nTregs that had been treated with the MHC I Ab. In sensitized and challenged mice depleted of NK cells, transfer of anti-MHC I-treated Tregs failed to down-regulate AHR or inflammation, or increase levels of IL-10 or TGF-β. Previously, we showed that host CD8 expression was essential for nTreg activation using $CD8^{-/-}$ mice (20, 21). In the present study, the role of CD8 in the host was demonstrated in WT recipients. Pretreatment of recipient mice with anti-CD8β intratracheally by microspray before the instillation of OVA-$CD4^+CD25^+$ nTregs also prevented their suppressive activities and the full development of lung allergic responses in these animals proceeded normally, accompanied by Th2 cytokine production and low levels of IL-10 and TGF-β. Therefore, in the lungs of allergen sensitized and challenged BALB/c or C57BL/6 mice, activation of nTregs and expression of their suppressive properties were dependent on interaction/engagement of MHC I and CD8 but did not involve Ag-specific TCRs.

In these studies of nTregs, little or no evidence demonstrating direct involvement or requirement for Ag-specific TCRs in the regulatory activities was shown. The importance of the Ag-specific TCRs described in earlier studies was primarily by inference (23–25). In some of the same studies, Ag specificity also appeared to be nonessential because transfer of total splenocytes and $CD4^+$ T cells from normal donors also provided effective suppression (25). Unlike Ag-induced Tregs, activation of the nTregs via an Ag-specific TCR may not be required under certain physiologic conditions as nTregs with no known Ag-specific TCRs were shown to be effective in suppressing lung allergic responses providing normal CD8-MHC I interactions were possible in recipient mice (20, 21). It is unlikely that any of the mechanisms involved in TCR repertoire selection played a role in our model because nTregs were isolated from naive donors and adoptively transferred into recipients at a time point in our protocol when active immunization to allergen was completed and insufficient time (96 h) would have elapsed between transfer and analysis of nTreg suppressive activity to have enabled TCR selection and expansion of Ag-specific Tregs. Further, supporting the lack of Ag specificity of nTregs, numbers of Ag-specific $CD4^+$ T cells in lymph nodes were reported to be near baseline levels at day 3 and only significantly increased at day 6 following active immunization (39). In addition, differential Ag-specific tetramer dissociation of $CD4^+$ T cells based on their TCR repertoire selection was only detected following primary sensitization at day 6 and secondary sensitization at day 102 (40). In a complicated system of bone marrow cell reconstitution in irradiated hosts, transferred $CD4^+CD25^+$ T cells were also shown to undergo transgenic TCR repertoire modification following active sensitization at day 18, a much longer elapsed time between sensitization and detection than in the present study (34).

Both production and release of IL-10 and TGF-β, and cell-to-cell contact, have been invoked as the mechanisms underlying these regulatory activities of Tregs (5–8). Previously, we demonstrated the critical role of IL-10 in the induction and release of TGF-β from nTregs which mediated the suppressive activities of nTregs and indeed, inhibition of TGF-β attenuated the suppressive activities (19). We suggest that the Ag-nonspecific suppression of lung allergic responses by nTregs is primarily cytokine-dependent, as evidenced by the increases in levels of IL-10 and TGF-β in BAL fluid of recipient mice given OVA-$CD4^+CD25^+$ but not OVA-$CD4^+CD25^-$ T cells. Cell-to-cell-dependent mechanisms may also be involved based on the requirements for CD8-MHC I interactions (20) and the findings that transferred $KJ1.26^+$ nTregs were identified in close proximity to host $CD8^+$ T cells in the airway lumens and lung parenchyma by immunohistochemical staining.

In summary, these in vitro and in vivo data demonstrate that the activation and expression of nTreg activity in the lung was not Ag-specific. Significant suppression of AHR, eosinophilic lung inflammation, Th2 cytokine production, and goblet cell metaplasia was observed in nTregs from WT mice, in mice expressing a transgene for a nonrelevant allergen, and in mice where the TCR was blocked by specific Ab. In each instance, suppression was accompanied by the up-regulation of IL-10 and TGF-β, and the data suggested close contacts in the lung between $CD8^+$ T cells and

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these nTregs. Controlling the activation of this subset of Tregs offers a novel therapeutic approach for the treatment of lung allergic diseases.

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Disclosures

The authors have no financial conflict of interest.

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