

Plasticity of Regulatory T Cells: Subversion of Suppressive Function and Conversion to Enhancement of Lung Allergic Responses^{1,2}

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Activation of CD4⁺CD25⁺Foxp3⁺ naturally occurring regulatory T cells (nTregs) resulting in suppression of lung allergic responses requires interaction of MHC class I on nTregs and CD8. In the absence of CD8 (CD8^{-/-} recipients), transferred nTregs restored airway hyperresponsiveness, eosinophilic inflammation, and IL-13 levels following allergen exposure. Enhancement of lung allergic responses was accompanied by reduced expression of Foxp3 and increased expression of IL-13 in the transferred nTregs. In CD8^{-/-} recipients pretreated with glucocorticoid-induced TNFR-related protein-ligand Ab, the transferred nTregs maintained high levels of Foxp3 and did not result in altered lung responses. Thus, the regulatory function of nTregs can be subverted by reducing the expression of Foxp3 and following signaling through glucocorticoid-induced TNFR-related protein are converted nTregs into IL-13-producing CD4⁺ T cells mediating lung allergic responses. *The Journal of Immunology*, 2008, 180: 7117–7124.

Naturally occurring CD4⁺CD25⁺ T cells (nTregs),⁴ a small (5–10%) subset of CD4⁺ T cells, play a major role in modulating the outcome of various diseases including autoimmunity, cancer, infection, transplantation, and allergy (1). They are defined phenotypically by the constitutive expression of the forkhead/winged helix transcription factor Foxp3 (2, 3), low-affinity IL-2R α -chain (CD25) (4), CTLA-4 (5), and glucocorticoid-induced TNFR-related protein (GITR) (6–8), and functionally by their ability to suppress the proliferation of naive CD4⁺CD25⁻ T cells in vitro (9, 10). Anergy is another unique feature of nTregs that has been shown to closely associate with suppressive activities (9).

Unlike nTregs, CD4⁺ T effector cells express CD25 only transiently, have marked proliferative capacity dividing rapidly, and do not up-regulate expression of Foxp3 upon activation. They have been shown to be an important source of proinflammatory IL-4, IL-5, and IL-13 and play a central role in the pathogenesis of asthma (11–13). Allergic asthma is associated with increased air-

way hyperresponsiveness (AHR) and inflammation, increased levels of Th2 cytokines, goblet cell metaplasia, excessive mucus production, elevated serum Ag-specific IgE, and structural remodeling of the airways (14, 15). Therapeutically, modulation of these processes can be prevented by depletion of the CD4⁺ cell population (16, 17) or by inhibition and/or alteration of their activities (18, 19) but, in contrast, removal of nTregs resulted in an enhancement of these changes (20). Accumulating evidence supports the pivotal role for these CD4⁺CD25⁺Foxp3⁺ T cells in the regulation of the development and outcome of allergic diseases in animals and humans (1, 3, 20–22). The suppressive activity of nTregs has been linked to a number of mechanisms, including the production and the release of the immunoregulatory cytokines IL-10 and TGF- β (1, 3, 21–23).

A number of factors control the development and function of these regulatory T cells (Tregs) including Foxp3, GITR, and GITR ligand (GITR-L) (2, 8, 28, 30–35). Foxp3 has been shown to be restricted to T regulatory cells and has an indispensable role in the differentiation of nTregs (36). Moreover, the expression of Foxp3 has been linked to the suppressive activity of these cells as demonstrated in disorders of Foxp3 expression (37), and where ectopic expression of a transgene encoding Foxp3 or a retroviral vector encoding Foxp3 results in the acquisition of suppressive properties by non-Tregs (38, 39). It appears that continuous expression of Foxp3 is needed to actively maintain the regulatory activity of these cells (40). A number of studies have suggested that under physiological conditions, the Foxp3-mediated Treg cell differentiation program is stable, with little reversibility (41), and that Foxp3 actively represses adoption of alternative CD4⁺ T cell lineage fates (40–42). Recently, Wan and Flavell (34) demonstrated that attenuation of endogenous Foxp3 gene expression results in a subversion of the suppressive function of Tregs, converting these cells into Th2 (IL-4)-producing effector cells in vitro and in vivo.

Unraveling the mechanisms that control the activation of nTregs is essential to exploit and manipulate their therapeutic potential. Previously, we defined the requirements for the interaction of MHC class I (MHC I) on CD4⁺CD25⁺ Tregs and CD8 in the host

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⁴ Abbreviations used in this paper: nTregs, naturally occurring CD4⁺CD25⁺ T cells; AHR, airway hyperresponsiveness; BAL, bronchoalveolar lavage; C_{dyn}, dynamic compliance; GITR, glucocorticoid-induced TNFR-related protein; MCh, methacholine; PAS, periodic acid-Schiff; R_L, lung resistance; WT, wild type; GITR-L, GITR ligand; MHC I, MHC class I; Treg, regulatory T cell.

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as one possible mechanism for activation of these regulatory suppressor cells resulting in immunomodulation (down-regulation) of allergic responses in the lung (22). Intratracheal administration of nTregs before allergen challenge in sensitized recipient mice resulted in a significant reduction in AHR and inflammation which were associated with a significant increase in the levels of IL-10 and TGF- β and concomitant decreases in the levels of IL-4, IL-5, and IL-13 in bronchoalveolar lavage (BAL) fluid (21, 22). These suppressive activities of nTregs could be abrogated by preventing the interaction between MHC I on the nTregs with host CD8 (22). In the present study, we investigated the subversion of suppressive function and the conversion of nTregs into CD4⁺ effector T cells resulting in the enhancement of lung allergic responses.

Materials and Methods

Animals

Pathogen-free, 8- to 10-wk-old female C57BL/6 mice were obtained from The Jackson Laboratories; IL-5^{-/-}, IL-13^{-/-}, and CD8^{-/-} mice were provided by Dr. P. Marrack (National Jewish Medical and Research Center, Denver, CO). All mice were maintained on an OVA-free diet. All protocols were approved by the Institutional Animal Care and Use Committee.

Sensitization

Sensitization was conducted by i.p. injection of 20 μ g of OVA (grade V; Sigma-Aldrich) emulsified in 2.25 mg of alum hydroxide (AlumImject; Pierce) in a total volume of 100 μ l on days 1 and 14. Sensitized and naive littermates received aerosol challenges for 20 min each day on 3 consecutive days (days 26, 27, and 28) with 1% OVA in PBS using an ultrasonic nebulizer (AeroSonic ultrasonic nebulizer, DeVilbiss) (21).

Cell preparation and culture

Lung CD4⁺CD25⁺ T cells from naive mice were isolated following collagenase digestion of lungs and enriched using nylon wool columns as previously described (21). Lymphocytes were further purified by CD4⁺CD25⁺ Treg MACS beads (Miltenyi Biotec), resulting in a purity of >95% CD4⁺CD25⁺ T cells, of which >95% were Foxp3⁺.

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

Adoptive transfer

Recipient mice received 5×10^5 isolated lung CD4⁺CD25⁺ or CD4⁺CD25⁻ T cells intratracheally in 50 μ l of PBS before allergen challenge. In some experiments, CD4⁺CD25⁺ T cells were labeled with CFSE or treated with anti-GITR mAb (200 μ g) (DTA-1; eBioscience) in vitro 1 h before transfer following extensive washing.

Antibodies

mAb from the culture supernatants of the IgG-producing hybridoma GK1.5 (anti-mouse CD4) was purified by protein G chromatography. Anti-mouse CD4 Ab (800 μ g) was injected i.v. 4 and 2 wk before allergen challenge. Anti-GITR-L was obtained from eBioscience. Mice were given anti-GITR-L (30 μ g) intratracheally using the Microsprayer (Penn-Century, Philadelphia, PA) just before adoptive transfer of nTregs.

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 (MCh) administered for 10 s (60 breaths/min, 500- μ l tidal volume) (21). Airway resistance (R_L) and dynamic compliance (C_{dyn}) were continuously computed (Labview; National Instruments) by fitting flow, volume, and pressure to an equation of motion. Maximum values of R_L and lowest C_{dyn} values were taken and expressed as a percentage change from baseline following PBS aerosol.

Bronchoalveolar lavage

Immediately following measurement of AHR, lungs were lavaged (1×1 ml, 37°C). Total leukocyte numbers were counted (Coulter Counter; Coulter). Differential cell counts were performed under light microscopy

by counting at least 200 cells on cytocentrifuged preparations (Cytospin 2; Cytospin), stained with Leukostat (Fisher Diagnostics), and differentiated by standard hematological procedures.

Determination of serum Ab titers by ELISA

Serum levels of total IgE, OVA-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 supernatants of in vitro-cultured lung cells were measured by ELISA (IL-4, IL-5, IL-10, IFN- γ , TGF- β from BD Biosciences/BD Pharmingen and IL-13 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, and 0.2% sodium azide), were labeled with the following conjugated Abs purchased from BD Pharmingen: anti-CD3 FITC, PE, PerCP, allophycocyanin (17A2); anti-CD4 FITC, PE, PerCP, allophycocyanin (L3T4); anti-CD25 FITC (7D4), PE (PC61); anti-CD8 α FITC, PE, PerCP (53-6.7); and anti-GITR PE (DTA-1). For intracellular staining, cells were stimulated with PMA (100 ng/ml) and ionomycin (2 μ g/ml; Sigma-Aldrich) in complete medium overnight and for 6 h in the presence of brefeldin A (10 μ g/ml; Sigma-Aldrich). Cells were fixed, permeabilized using an eBioscience Fixation/Permeabilization Kit, and stained with anti-IL-10 PE and anti-TGF- β PE (BD Pharmingen), IL-13 (R&D Systems), and Foxp3 PE (eBioscience). Stained cells were analyzed on a FACSCalibur (BD Biosciences) using CellQuest software. Fluorescence intensity was compared with negative controls and cells were incubated with PE-coupled streptavidin alone. Nonviable cells were excluded from the analysis by staining with 7-aminoactinomycin D (eBioscience).

Histochemistry

Lungs were fixed by inflation (1 ml) and immersion in 10% formalin. For detection of mucus-containing cells in formalin-fixed airway tissue, sections were stained with periodic acid-Schiff (PAS) and quantitated as previously described (24).

Statistical analysis

ANOVA was used to determine the levels of difference between all groups. Comparisons for all pairs were performed using the Tukey-Kramer honest significant difference test. The *p* values for significance were set to 0.05. Values for all measurements were expressed as the mean $\pm$ SEM.

Results

CD4⁺CD25⁺ T cells enhance AHR and inflammation

The aim of these experiments was to investigate the regulatory capacity of naturally occurring CD4⁺CD25⁺Foxp3⁺ T cells (nTregs) isolated from the lungs of naive wild-type (WT) donor mice on airway responsiveness and eosinophilic inflammation in sensitized and challenged CD8^{-/-} and WT mice. As shown in Fig. 1, *A* and *B*, sensitized and challenged CD8^{-/-} mice failed to develop significant increases in lung resistance and corresponding decreases in dynamic compliance; in contrast, sensitized and challenged WT mice developed significant increases in R_L and decreases in C_{dyn} throughout the MCh dose response, consistent with published reports (21, 22). nTregs were previously shown to suppress CD8⁺ T cell-mediated lung allergic responses in CD8 α ^{+/+} and CD8 α ^{-/-} mice reconstituted with negatively selected, primed CD8⁺ T cells (22). Surprisingly, in the absence of recipient CD8, intratracheal administration of nTregs had an opposite effect to that seen in WT mice, restoring AHR in sensitized and challenged (but not nonsensitized; data not shown) CD8^{-/-} recipients (Fig. 1, *A* and *B*). In WT mice, transfer of these nTregs maintained suppressive activity, significantly reducing AHR.

Following sensitization and allergen challenge, CD8^{-/-} mice developed a modest increase in the number of BAL eosinophils but

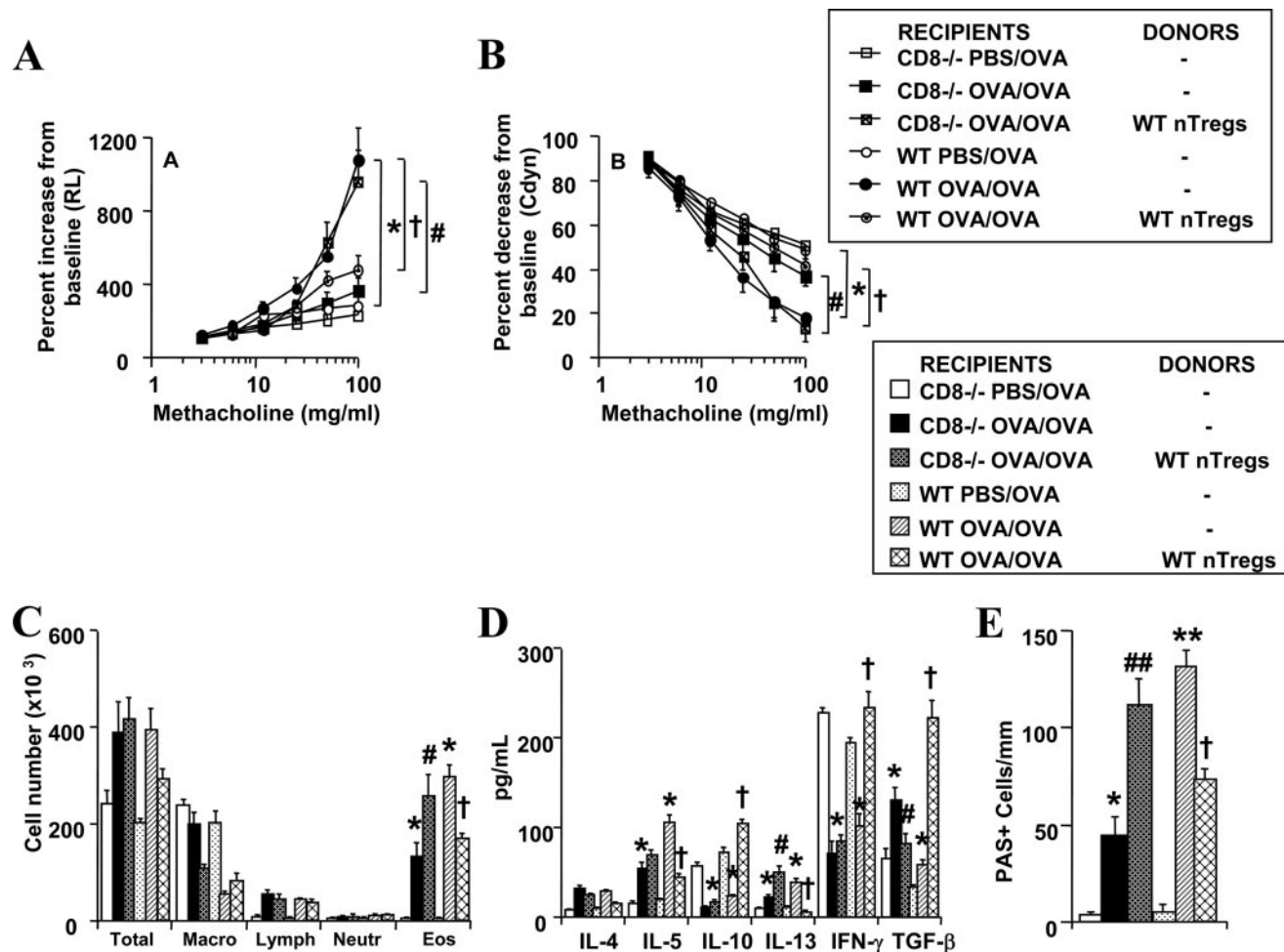


FIGURE 1. Effect of transfer of nTregs from naive WT donors into sensitized and challenged CD8^{-/-} and WT recipients. Sensitized CD8^{-/-} and WT mice received nTregs before challenge. *A* and *B*, AHR; *C*, BAL fluid inflammatory cell composition; *D*, BAL cytokine levels; and *E*, number of PAS⁺ cells. Shown are the means $\pm$ SEM from three independent experiments (four mice per group, $n = 12$ mice analyzed for each group). *, $p < 0.05$ and **, $p < 0.01$ comparing sensitized and challenged mice (OVA/OVA) to mice challenged alone (PBS/OVA). #, $p < 0.05$ and ##, $p < 0.01$ comparing the enhancing activity in CD8^{-/-} recipients of nTregs compared with CD8^{-/-} mice. †, $p < 0.05$ comparing the suppressive activity in WT recipients of nTregs compared with WT mice.

significantly less than sensitized and challenged WT mice as shown in Fig. 1C. Associated with the enhancement of AHR in CD8^{-/-} mice, following intratracheal transfer of nTregs, a significant increase in BAL eosinophils, comparable to sensitized and challenged WT mice, was detected in the CD8^{-/-} mice. By contrast, airway eosinophilia in sensitized and challenged WT mice was significantly reduced following transfer of nTregs.

BAL cytokines levels following adoptive transfer of nTregs

Following sensitization and allergen challenge, but not challenge alone, reduced levels of IL-10 and IFN- γ and modest increases in the levels of IL-4, IL-5 but not IL-13 were detected in the BAL of CD8^{-/-} mice (Fig. 1D). Consistent with previous reports (21, 22), a marked increase in BAL TGF- β levels was also detected in these mice. In contrast to WT mice given nTregs where levels of IL-4, IL-5, and IL-13 were significantly reduced and IL-10 and TGF- β increased, intratracheal administration of nTregs into sensitized and challenged CD8^{-/-} recipients resulted in significant increases in the levels of IL-5 and IL-13 and decreases in the levels of IL-10 and TGF- β .

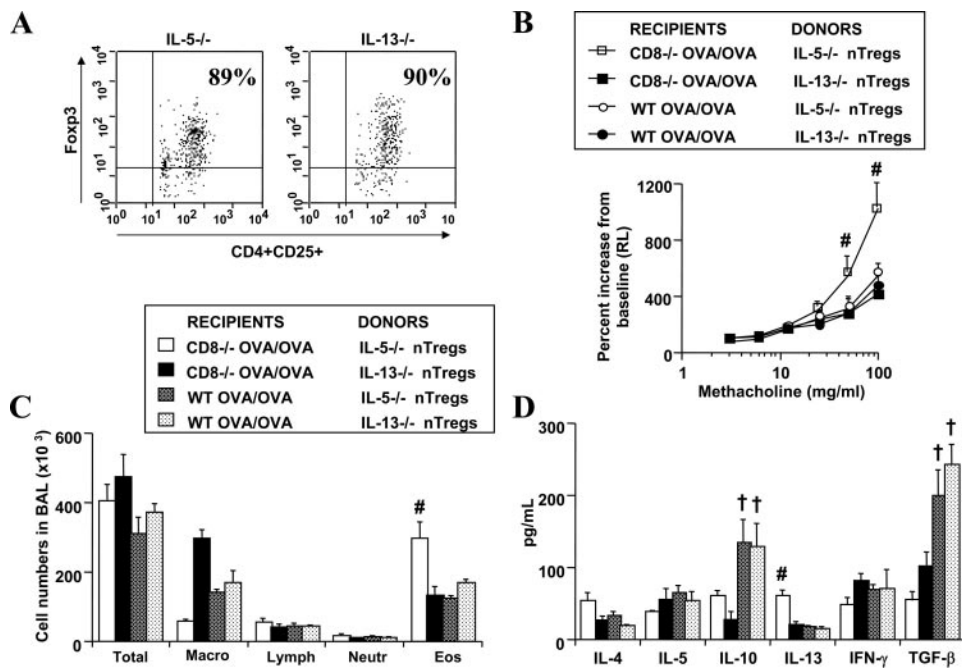
Levels of allergen-specific Abs are unaffected by adoptive transfer of nTregs

Allergen sensitization and challenge was associated with increases in total IgE and allergen-specific IgE, IgG1, IgG2a, and IgG2b levels in the serum. Intratracheal transfer of nTregs did not alter the levels of total IgE or allergen-specific Abs in the sera of either WT or CD8^{-/-} recipient mice (data not shown).

Number of PAS⁺ cells are reduced following transfer of nTregs

Allergen sensitization and challenge are associated with significant changes in lung histopathology, including mucus hyperproduction and goblet cell metaplasia. These changes were significantly reduced following the transfer of nTregs in WT mice. As previously reported (25), sensitized and challenged CD8^{-/-} mice, but not challenged alone animals, developed these characteristic changes, including increases in PAS⁺ cells but at levels significantly less than WT mice (Fig. 1E). In contrast to WT mice, the number of PAS⁺ cells in CD8^{-/-} recipients was significantly increased following the transfer of nTregs.

FIGURE 2. Requirement for IL-5 or IL-13 in nTreg activity. CD4⁺CD25⁺ T cells isolated from IL-5^{-/-} or IL-13^{-/-} donors were administered to sensitized CD8^{-/-} and WT recipients before allergen challenge. **A**, Representative Foxp3 staining of isolated CD4⁺CD25⁺ T cells from IL-5^{-/-} and IL-13^{-/-} donors; results from three separate experiments showed 91 ± 5.1% and 90 ± 7.4% Foxp3⁺ cells, respectively. **B**, AHR to inhaled MCh; **C**, BAL cell composition; and **D**, BAL cytokine levels. Results are shown as means ± SEM from three independent experiments (*n* = 12). #, *p* < 0.05 comparing the enhancing activity in CD8^{-/-} recipients of IL-5^{-/-} nTregs compared with other groups. †, *p* < 0.05 comparing the suppressive activity in WT recipients of IL-5^{-/-} or IL-13^{-/-} nTregs compared with other groups. Macro, Macrophages; Lymph, lymphocytes; Neutr, neutrophils; Eos, eosinophils.



Taken together, the data identify the loss of regulatory activities of nTregs in the absence of CD8 in the host, resulting in turn, in enhancement of AHR, eosinophilic airway inflammation, levels of Th2 cytokines, and mucus hyperproduction. Moreover, in each instance the enhancement was restricted to transfer of CD4⁺CD25⁺ T cells; transfer of CD4⁺CD25⁻ T cells was without effect in these CD8^{-/-} recipients (data not shown).

IL-5^{-/-} but not IL-13^{-/-} CD4⁺CD25⁺ T cells augment AHR and inflammation

The proinflammatory cytokines IL-5 and IL-13 have been identified as critical to the development of airway eosinophilia and mucus production in allergen-induced lung responses (24, 26, 27). To investigate their involvement in the enhancement of lung allergic responses in sensitized and challenged CD8^{-/-} mice, we investigated the consequences of intratracheal administration of nTregs isolated from naive IL-5^{-/-} or IL-13^{-/-} donors. More than 95% of isolated nTregs from these mice constitutively expressed Foxp3 as in WT mice (Fig. 2). nTregs isolated from IL-5^{-/-} but not IL-13^{-/-} mice induced a significant enhancement of AHR and the number of BAL eosinophils (Fig. 2, B and C), similar to the responses seen with transfer of WT nTregs (Fig. 1, A and B). In contrast, CD4⁺CD25⁺ T cells from both IL-5^{-/-} and IL-13^{-/-} mice maintained their suppressive activities (Fig. 2, B and C), lowering AHR and airway inflammation in WT recipients, similar to transfer of WT nTregs (Fig. 1).

Intratracheal transfer of IL-5^{-/-} nTregs, similar to transfer of WT nTregs (Fig. 1), resulted in significantly higher levels of IL-13 and lower levels of IL-10 and TGF-β in the BAL of CD8^{-/-} recipients (Fig. 2D). In contrast, the levels of IL-13 failed to increase following the transfer of nTregs from IL-13^{-/-} donors into these recipients. Interestingly, nTregs from both IL-5^{-/-} and IL-13^{-/-}, similar to WT nTregs, consistently lowered the levels of IL-4, IL-5, and IL-13 and significantly increased the levels of IL-10 and TGF-β in the BAL of WT recipients. These data demonstrated that IL-13 was required for the nTreg-mediated enhancement of lung allergic responses in CD8^{-/-} recipients but not for the suppression in WT recipients.

Depletion of CD4⁺ T cells in sensitized and challenged CD8^{-/-} mice fails to alter nTreg enhancement of lung allergic responses

We next investigated the capacity of nTregs to enhance lung allergic responses independently of recipient CD4⁺ T cells. Recipient CD4⁺ T cells were depleted by anti-CD4 administration at 4 and 2 wk before the transfer of nTregs, resulting in <1–2% remaining CD4⁺ T cells in the lung and peribronchial lymph nodes (Table I). In sensitized and challenged WT recipients, adoptive transfer of nTregs from IL-13^{+/+} donors suppressed AHR and eosinophilic airway inflammation, regardless of the depletion of recipient CD4⁺ T cells (Fig. 3, A and B). Despite depletion of CD4⁺ cells in sensitized and challenged CD8^{-/-} recipients, full enhancement of AHR and inflammation developed following transfer of nTregs from IL-13^{+/+} donors (but not IL-13^{-/-} donors), suggesting that the (donor) nTregs were the primary effector cells mediating either suppression or enhancement, independently of recipient CD4⁺ T cells.

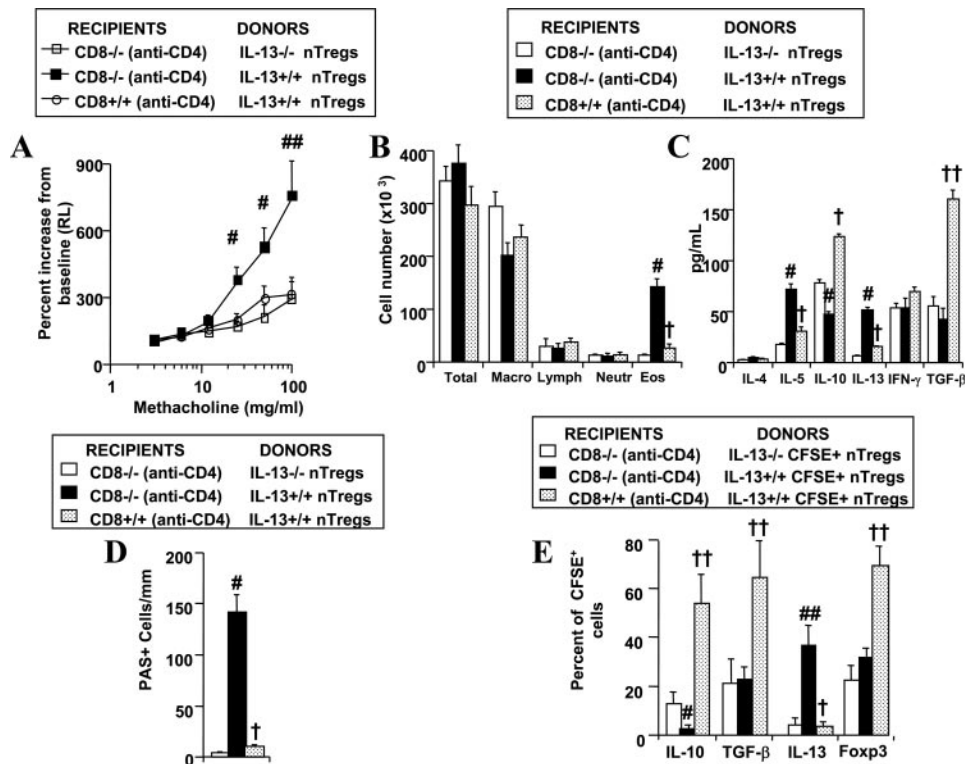
Intratracheal administration of nTregs from IL-13^{+/+} donors increased the levels of IL-13 in the CD8^{-/-} recipients depleted of CD4⁺ cells (Fig. 3C). This was not seen following transfer of nTregs from IL-13^{-/-} donors. In WT recipients, depletion of recipient CD4⁺ T cells reduced the levels of IL-5 and IL-13 significantly more than in mice which were not depleted and

Table I. CD4⁺ T cell depletion in lung and peribronchial lymph nodes

	CD3 ⁺ CD4 ⁺ Cells	
	Lung	Peribronchial Lymph Nodes
OVA/OVA (rat IgG)	1.57 × 10 ⁶ ± 0.07	1.4 × 10 ⁶ ± 0.1
OVA/OVA (anti-CD4)	1.60 × 10 ⁴ ± 0.04	2.1 × 10 ⁴ ± 0.03

C57BL/6 mice were sensitized and challenged with OVA. Anti-CD4 was injected i.v. 2 and 4 wk prior to allergen challenge. Single-cell preparations were prepared from lungs and peribronchial lymph nodes. Mononuclear cells were isolated and stained with CD3⁺ and CD4⁺ mAb. Absolute numbers were obtained. Results are from three separate preparations.

FIGURE 3. Effect of CD4⁺ T cell depletion in recipient mice on nTreg activity. Sensitized CD8^{-/-} and WT recipients were administered anti-CD4 Ab before transfer of nTregs and challenge from IL-13^{-/-} or WT donors. A, AHR; B, BAL cell composition; C, BAL cytokine levels; D, number of PAS⁺ cells; E, nTregs were labeled with CFSE before transfer, isolated after challenge, and intracytoplasmic staining was conducted on recovered CFSE⁺CD4⁺ cells from the lungs of recipient mice. The number of cells staining positive were calculated as a percentage of the total CFSE⁺ population. Results are shown as means $\pm$ SEM from three independent experiments ($n = 12$). #, $p < 0.05$ and ##, $p < 0.01$ comparing the enhancing activity in CD8^{-/-} recipients of IL-13^{+/+} nTregs compared with other groups. †, $p < 0.05$ and ††, $p < 0.01$ comparing the suppressive activity in WT recipients of IL-13^{+/+} nTregs compared with other groups.



received nTregs, and the levels of IL-10 and TGF- β remained significantly elevated following the transfer of nTregs from IL-13^{+/+} donors. The increased levels of IL-13 in CD8^{-/-} recipients receiving nTregs from IL-13^{+/+} but not from IL-13^{-/-} donors correlated with the higher number of PAS⁺ cells in the airways (Fig. 3D).

nTregs express increased IL-13 and decreased Foxp3 following transfer into sensitized and challenged CD8^{-/-} recipients

In the absence of recipient expression of CD8, the *in vivo* results suggested that transferred nTregs converted into potent effectors, acquiring a different phenotype with production of IL-13 instead of IL-10 and TGF- β . To track this possibility, nTregs were stained with CFSE before transfer and we monitored conversion by intracellular staining of isolated lung CFSE⁺CD4⁺ T cells 4 days later following incubation with PMA and ionomycin. Equal numbers of CFSE⁺CD4⁺ T cells were isolated from WT and CD8^{-/-} recipients. Fig. 3E illustrates the fraction of CFSE⁺CD4⁺ cells that stained positively for IL-10, IL-13, Foxp3, or TGF- β . As can be seen in CD4⁺ T cell-depleted WT recipients of WT nTregs, a high proportion of the isolated CFSE⁺CD4⁺ T cells were positive for IL-10, TGF- β , and Foxp3; few cells were IL-13⁺. In contrast, staining of IL-10, Foxp3, and TGF- β in CFSE⁺CD4⁺ T cells isolated from CD8^{-/-} recipients of either WT or IL-13^{-/-} nTregs was significantly lower. In these CD8^{-/-} mice, the decrease in expression of Foxp3 was associated with a corresponding increase in staining for IL-13 in CFSE⁺CD4⁺ T cells in recipients of WT but not IL-13^{-/-} nTregs.

These data demonstrated that in the absence of recipient CD8, the *in vivo* regulatory function of nTregs can be subverted from a suppressive phenotype and converted to an effector phenotype with reduced expression of Foxp3 and increased production of IL-13.

Anti-GITR-L but not anti-GITR pretreatment prevents conversion of nTregs in sensitized and challenged CD8^{-/-} mice

Both administration of GITR agonist Ab and increased GITR-L expression have been shown to nullify the suppressive activity of nTregs (8, 28, 29). Because only CD4⁺CD25⁺ but not CD4⁺CD25⁻ cells constitutively expressed high levels of GITR (Fig. 4A), we investigated the *in vitro* effects of anti-GITR and *in vivo* effects of anti-GITR-L on the functional conversion of lung nTregs obtained from WT donors. *In vitro* incubation of isolated WT lung nTregs with an agonistic anti-GITR mAb before transfer abrogated the suppression of AHR and inflammation in sensitized and challenged WT recipient mice (Fig. 4, B–D); however, this *in vitro* Ab treatment of nTregs did not influence the enhanced responses observed in CD8^{-/-} recipients. In parallel to the loss of suppression of AHR and airway eosinophilia following pretreatment of WT nTregs with anti-GITR in WT recipients, sensitization and challenge resulted in increased levels of IL-13 in BAL (Fig. 4D) and the number of PAS⁺ cells (Fig. 4E), similar to levels seen in sensitized and challenged WT mice that did not receive nTregs.

In contrast, treatment of CD8^{-/-} recipients with a mAb to GITR-L before transfer of nTregs prevented the enhancement of AHR and eosinophilic inflammation (Fig. 4, B–D). Furthermore, administration of anti-GITR-L to CD8^{-/-} mice before transfer of WT nTregs prevented the increases in levels of IL-13 (Fig. 4D) and associated increases in the number of PAS⁺ cells in the airways (Fig. 4E), supporting the importance of GITR-L in the functional conversion of nTregs in CD8^{-/-} recipients. In WT mice given nTregs, anti-GITR-L treatment had little effect on AHR, eosinophilic inflammation, or the levels of IL-13 and number of PAS⁺ cells as all remained suppressed (Fig. 4, B–F). Treatment of CD8^{-/-} mice or WT mice with anti-GITR-L, without transfer of nTregs, did not affect development of AHR or any of the other responses (data not shown).

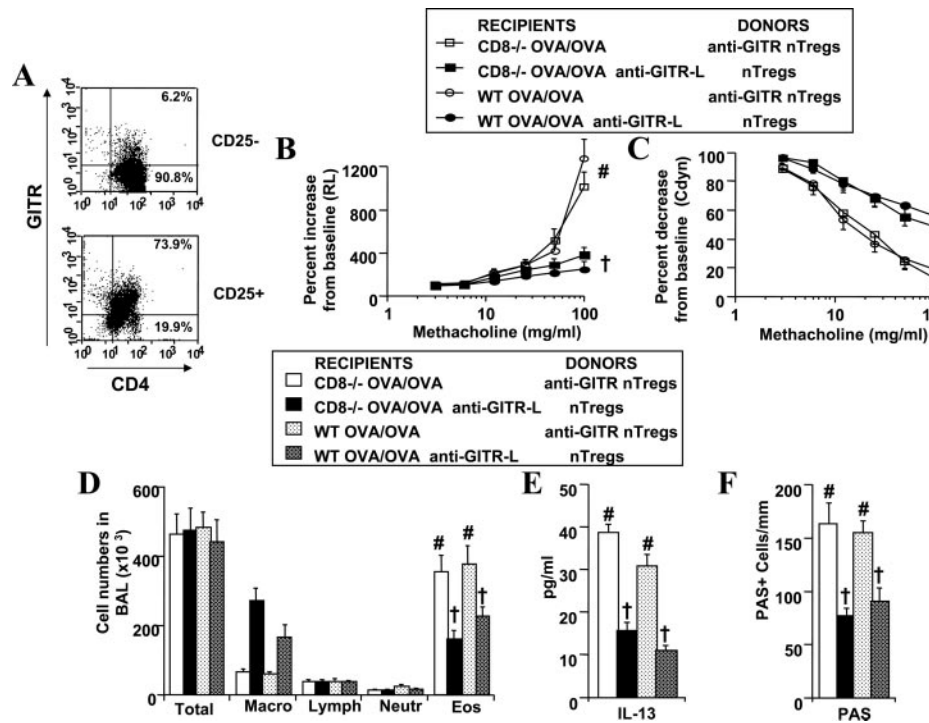


FIGURE 4. Effect of in vitro anti-GITR and in vivo anti-GITR-L treatment on lung allergic responses in sensitized and challenged recipients. Isolated nTregs from WT donors were treated with anti-GITR Ab in vitro for 1 h before transfer into sensitized and challenged CD8^{-/-} or WT recipients. In vivo, anti-GITR-L (30 μ g) was administered intratracheally to sensitized and challenged WT and CD8^{-/-} mice before transfer of nTregs. **A**, Surface staining of GITR on CD4⁺CD25⁺ or CD4⁺CD25⁻ T cells. **B** and **C**, AHR; **D**, BAL cell composition; **E**, BAL cytokine levels; and **F**, number of PAS⁺ cells. Results represent means $\pm$ SEM from three independent experiments ($n = 12$). #, $p < 0.05$ comparing the enhancing activity in CD8^{-/-} or WT recipients of anti-GITR-treated nTregs compared with other groups. †, $p < 0.05$ comparing the suppressive activity in CD8^{-/-} or WT recipients of nTregs treated in vivo with anti-GITR-L to other groups. Macro, Macrophage; Lymph, lymphocyte; Neutr, neutrophil; Eos, eosinophil.

Discussion

Naturally arising CD4⁺CD25⁺Foxp3⁺ Tregs are pivotal cells in the maintenance of self-tolerance and in the context of allergen-induced responses in the lung are active suppressors of these responses including the development of AHR, eosinophilic airway inflammation, Th2 cytokine production, goblet cell metaplasia, and mucus hyperproduction. In earlier studies, we demonstrated that the in vivo functional activation of nTregs resulting in suppression of lung allergic responses was dependent on the interaction of MHC I on the nTregs and CD8 in the host (22). Any interference with this engagement, either by gene knockout or Ab blocking resulted in loss of suppression and prevented the production and release of IL-10 and TGF- β , which were shown to be important for the regulatory activity of nTregs (21).

In the present study, we demonstrated that transfer of isolated lung nTregs from WT mice exhibited different functions dependent on the recipient expression of CD8. In WT recipients, adoptive transfer of nTregs fully suppressed all aspects of the allergen-induced airway responses. However, when transferred into CD8-deficient mice, these same nTregs restored AHR, airway eosinophilia, and goblet cell metaplasia to the same levels seen in sensitized and challenged WT mice. Thus, the same population of cells was shown to have different functional properties depending on the milieu in the recipient mice. Based on the phenotypic characteristics of the cells (>95% Foxp3⁺), it is assumed that both the suppressive and enhancing activities were restricted to the same population of transferred CD4⁺CD25⁺ T cells since these activities were not seen following transfer of CD4⁺CD25⁻ cells.

Despite sensitization and challenge with allergen, CD8^{-/-} mice, unlike WT mice, develop limited AHR and eosinophilic

airway inflammation. Associated with this hyporesponsiveness, levels of the proallergic cytokines IL-4, IL-5, and IL-13 were only modestly increased or unchanged. However, the cytokine profile in BAL fluid was altered dramatically following intratracheal administration of nTregs but not non-Tregs (CD4⁺CD25⁻) isolated from the lungs of naive WT mice. Specifically, levels of IL-5 and IL-13 increased significantly to levels comparable to sensitized and challenged WT mice, and this increase was associated with a significant enhancement (normalization) of all lung allergic responses, including AHR, airway inflammation, and number of mucus-producing cells. In contrast, transfer of WT nTregs suppressed AHR and inflammation in sensitized and challenged WT hosts by reducing the levels of proallergic cytokines IL-4, IL-5, and IL-13 while increasing the levels of IL-10 and TGF- β , consistent with previous reports and central to the suppressive activity of nTregs (22, 23). To identify the role of IL-5 and IL-13 in the enhancement (or suppression) of lung allergic responses, we assessed the effects of nTregs isolated from IL-5^{-/-} or IL-13^{-/-} mice. In CD8^{-/-} recipients, nTregs from IL-13^{-/-} mice failed to enhance the responses although they were fully suppressive in WT recipients including the induction of IL-10 and TGF- β . Correlating with the enhancement of allergic responses in CD8^{-/-} recipients, a significant increase in levels of IL-13 in BAL fluid and PAS⁺ cells in the airways was detected following the transfer of nTregs from WT and IL-5^{-/-} but not IL-13^{-/-} mice.

It has been suggested that converted nTregs can influence Th2 responses in neighboring cells (34). To determine whether or not the transferred nTregs were directly responsible for enhancing the responses in CD8^{-/-} recipients and whether or not host cells were involved, recipient mice were depleted of CD4⁺ T cells

before intratracheal transfer of nTregs. The results indicated that despite depletion of recipient CD4⁺ T cells in sensitized and challenged CD8^{-/-} recipients, the transferred nTregs (from IL-13^{+/+} donors) still resulted in the enhancement of AHR, eosinophilic inflammation, and IL-13 production.

Labeling of nTregs with CFSE before transfer enabled their recovery from the lungs of sensitized and challenged CD4⁺ T cell-depleted recipients. Although equal numbers of transferred CFSE⁺CD4⁺ cells were isolated from WT and CD8^{-/-} recipients, analysis of intracellular cytokine and Foxp3 expression revealed marked differences between CFSE⁺CD4⁺ cells recovered from CD8^{-/-} recipients and those recovered from WT recipients. Although a large proportion of CFSE⁺ nTregs recovered from WT recipients expressed Foxp3 and stained for IL-10 and TGF- β , with little to no expression of IL-13, a large proportion of the CFSE⁺ nTregs recovered from the CD8^{-/-} recipients expressed IL-13, with fewer positive cells for Foxp3, IL-10, or TGF- β . These data identified the association between suppressive activity and expression of Foxp3, while enhancing activity was associated with reduced Foxp3 activity. These results suggested that nTregs may not be fixed in their differentiation fate, but exhibit some plasticity to exogenous signals.

In addition to the controlling features of Foxp3, recent reports have demonstrated that GITR and GITR-L may influence the activity of Tregs. In different systems, incubation of Tregs with the agonistic GITR mAb DTA-1 increased expression of GITR-L, attenuated Treg-mediated suppression, and with additional stimulation could break anergy (28–30, 43). Exploiting these findings and the demonstration that CD4⁺CD25⁺ but not CD4⁺CD25⁻ T cells constitutively express high levels of GITR, we investigated the effects of anti-GITR (DTA-1) and anti-GITR-L on the functional subversion/conversion of nTregs. These data confirmed the reported in vitro findings and further defined a novel role for GITR-L engagement in the enhancement of lung allergic responses. In the absence of MHC I-CD8 interaction, in vitro treatment of nTregs with agonistic anti-GITR did not influence the outcome, with full enhancement seen in the CD8^{-/-} recipients. In contrast, when transferred into WT recipients, following in vitro treatment with anti-GITR, nTregs no longer suppressed AHR or airway inflammation. It is unclear how the GITR pathway attenuates the suppressive phenotype or how it may modulate Foxp3 expression; signaling via GITR is known to activate NF- κ B (28). In vitro treatment before intratracheal transfer may have initiated a cascade of costimulatory signals in nTregs that exceeded or counteracted signals that might subsequently have been triggered in vivo through MHC I-CD8 interactions. The data also confirm that suppression of lung allergic responses in WT recipients by transferred Ab-treated nTregs was not the result of their removal by opsonization because of the positive enhancement with these same cells observed in CD8^{-/-} recipients. Stephens et al. (44) suggested that ligation of GITR on responder T cells but not Tregs was required to abrogate suppression. Our data do not support the assertion of promoting resistance in responder cells by GITR signaling, since in our model recipient mice were never treated with the Ab, only nTregs before transfer. The importance of GITR in regulating the suppressor phenotype was complemented by defining the role of GITR-L in the enhancer phenotype. When CD8^{-/-} recipients were treated (in vivo) with Ab to GITR-L before transfer of WT nTregs, the enhancement of all lung allergic responses, including AHR, airway eosinophilia, IL-13, and mucus-containing goblet cells, was lost. The loss of enhancing activity was not due to the elimination of

these cells in vivo because nTregs treated with anti-GITR-L still maintained their suppressive activity in WT recipients.

These in vitro and in vivo data identify for the first time a novel mechanism underlying the subversion of the regulatory functions of naturally occurring CD4⁺CD25⁺ T cells by reducing the expression of Foxp3, converting these cells into Th2 effector cells producing IL-13, and mediating lung allergic responses. In the absence of functional activation through the engagement of MHC I and CD8, GITR-L signaling acts directly on nTregs, effectively subverting their regulatory function and converting them into effector cells which by themselves are sufficient to mediate AHR, eosinophilic lung inflammation, Th2 cytokine production, and goblet cell metaplasia.

Together, these data identify a “physiological” plasticity to the differentiation fate of nTregs in the context of lung allergic responses. In the presence of MHC I-CD8 interactions, isolated CD4⁺CD25⁺Foxp3⁺ lung nTregs are actively suppressive of these responses in association with production of IL-10 and TGF- β . In the absence of MHC I-CD8 interactions, these same nTregs in vivo express decreased Foxp3 (and IL-10 and TGF- β), while becoming potent IL-13 producers, leading to enhancement of lung allergic responses. Thus, in addition to MHC I-CD8 interactions, GITR-GITR-L interactions also serve to control the differentiation fate of these nTregs in vivo. Agonistic signaling through GITR appears to down-regulate suppressive activity while regulatory GITR-L blockade in vivo controls expression of the enhancing phenotype. The hierarchy of these regulatory pathways need further definition but the data suggest that they can be manipulated in ways that may offer novel therapeutic strategies in asthma and potentially other inflammatory diseases.

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Disclosures

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