

Inducible and naturally occurring regulatory T cells enhance lung allergic responses through divergent transcriptional pathways

Anthony Joetham, BS,^a Michaela Schedel, PhD,^a Brian P. O'Connor, PhD,^b Soohyun Kim, PhD,^a Katsuyuki Takeda, MD, PhD,^a Jordan Abbott, MD,^a and Erwin W. Gelfand, MD^a *Denver, Colo*

Background: Regulatory T cells attenuate development of asthma in wild-type (WT) mice, with both naturally occurring regulatory T (nTreg) cells and inducible regulatory T (iTreg) cells exhibiting suppressive activity. When transferred into CD8-deficient (CD8^{-/-}) recipients, both cell types enhanced development of allergen-induced airway hyperresponsiveness. **Objective:** We sought to determine whether the pathways leading to enhancement of lung allergic responses by transferred nTreg and iTreg cells differed. **Methods:** nTreg cells (CD4⁺CD25⁺) were isolated from WT mice and iTreg cells were generated from WT CD4⁺CD25⁻ T cells after activation in the presence of TGF- β and transferred into sensitized CD8^{-/-} recipients before challenge. Development of airway hyperresponsiveness, cytokine levels, and airway inflammation were monitored. **Results:** Transfer of nTreg cells enhanced lung allergic responses, as did transfer of iTreg cells. Although anti-IL-13 reduced nTreg cell-mediated enhancement, it was ineffective in iTreg cell-mediated enhancement; conversely, anti-IL-17, but not anti-IL-13, attenuated the enhancement by iTreg cells. Recovered iTreg cells from the lungs of CD8^{-/-} recipients were capable of IL-17 production and expressed high levels of signature genes of the T_H17 pathway, *ROR γ t* and *Il17*, whereas reduced expression of the Treg cell key transcription factor forkhead box p3 (*Foxp3*) was observed. *In vitro* exogenous IL-6-induced IL-17 production in iTreg cells, and *in vivo* conversion of transferred iTreg cells was dependent on recipient IL-6. **Conclusions:** iTreg cells, similar to nTreg cells, exhibit functional plasticity and can be converted from suppressor cells to pathogenic effector cells, enhancing lung allergic responses, but these effects were mediated through different pathways. (J Allergy Clin Immunol 2016;■■■:■■■-■■■.)

Key words: Inducible and naturally occurring regulatory T cells, suppression, enhancement, asthma, IL-17, IL-13

Abbreviations used

AHR:	Airway hyperresponsiveness
APC:	Allophycocyanin
BAL:	Bronchoalveolar lavage
FACS:	Fluorescence-activated cell signaling
FITC:	Fluorescein isothiocyanate
Foxp3:	Forkhead box p3
GITR:	Glucocorticoid-induced TNF receptor-regulated protein
GITRL:	Glucocorticoid-induced TNF receptor-regulated protein ligand
iTreg:	Inducible regulatory T
MCh:	Methacholine
nTreg:	Naturally occurring regulatory T
PE:	Phycoerythrin
PerCP:	Peridinin-chlorophyll-protein complex
OVA:	Ovalbumin
R _L :	Lung resistance
RT-PCR:	Real-time PCR
WT:	Wild-type

Thymus-derived naturally occurring CD4⁺CD25⁺ regulatory T (nTreg) cells, which are characterized by constitutive expression of the transcription factor forkhead box p3 (Foxp3), are critical in immune tolerance and homeostasis. Defects in their function, numbers, or both resulted in immunologic imbalance and autoimmunity, as observed in patients with X-linked immune dysregulation, polyendocrinopathy, and enteropathy syndrome¹ and Scurfy mice.² Other regulatory T cells have been described,³ including CD4⁺CD25⁻ T cells, which acquire regulatory function and phenotype by expressing Foxp3 in nonthymic tissue and *in vitro* after culture with TGF- β .^{4,5} The immunomodulatory activities of these distinct subpopulations might be complementary in maintaining immune homeostasis and overlap, although with differing efficiencies, reflecting differences in developmental requirements,³ activation,^{6,7} and functional stability.⁸⁻¹⁰

In both human subjects and animals, allergic asthma is an inflammatory disease of the airways characterized by increases in airway hyperresponsiveness (AHR) and inflammation, type 2 cytokine skewing, goblet cell metaplasia, excessive mucus production, increased antigen-specific IgE levels, and structural remodeling of the airways. Both nTreg cells and inducible regulatory T (iTreg) cells have been shown to be effective regulators of lung responses after allergen sensitization and challenge.¹¹ In part, these suppressive activities were linked to IL-10 and TGF- β released from regulatory T cells^{12,13} in both an antigen-specific¹⁴ and antigen-nonspecific manner.^{15,16} Interestingly, these suppressive activities were demonstrated after adoptive transfer into wild-type (WT) recipients^{10,16} but not in

From ^athe Division of Cell Biology, Department of Pediatrics, and ^bthe Center for Genes, Environment & Health, National Jewish Health.

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Corresponding author: Erwin W. Gelfand, MD, Department of Pediatrics, National Jewish Health, 1400 Jackson St, Denver, CO 80206. E-mail: gelfande@njhealth.org. 0091-6749/\$36.00

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CD8-deficient (CD8^{-/-}) recipients. In CD8^{-/-} recipients these same nTreg cells were shown to be capable of converting *in vivo* into pathogenic IL-13-producing effector T cells, enhancing the full spectrum of lung allergic responses. This enhancement was modulated by the glucocorticoid-induced TNF receptor-related protein (GITR)-dependent activation of c-Jun N-terminal kinase 2.⁸⁻¹⁰ Direct interactions between CD8⁺ T cells and nTreg cells were demonstrated¹⁷ and shown to be necessary for expression of suppressor activity⁶ and development of regulatory activities.^{12,16} In the absence of CD8 (CD8^{-/-} mice) or after antibody-mediated depletion of CD8⁺ T cells, the suppressive function of CD4⁺CD25⁺ T cells was attenuated and Foxp3 levels were reduced, as was the production of IL-10 and TGF- β .^{6,8} In contrast, IL-6 levels in these cells were markedly increased.¹⁸ In CD8^{-/-} mice the loss of suppression was not terminally fixed because reconstitution (through transfer of CD8⁺ T cells) of CD8^{-/-} mice restored the regulatory function and phenotype of nTreg cells, suggesting that reprogramming remained possible.¹⁸

It is now evident that several subsets of T cells with similar phenotypes are capable of regulating the development of lung allergic responses. The functional fidelity of nTreg cells has been investigated and illustrated a plasticity that was dependent on the integration of signals from the local cytokine environment, stimulatory factors, and cell-to-cell interactions. Both loss of regulatory function and concomitant gain of effector function under certain inflammatory conditions^{8,19,20} and loss of suppression without apparent gain of effector function after stimulation with a GITR agonist antibody,⁸ glucocorticoid-induced TNF receptor-regulated protein ligand (GITRL),^{9,10} and IL-6^{18,21,22} have been reported for nTreg cells. In contrast, iTreg cells (CD4⁺CD25⁻ T cells differentiated in the presence of TGF- β) have been less well studied in terms of their functional plasticity.

In the present study we compared the regulatory and effector functions of nTreg and iTreg cells. Both subsets effectively suppressed the development of lung allergic responses when transferred into sensitized and challenged WT mice. In contrast, when transferred into sensitized and challenged CD8^{-/-} recipients, both subsets triggered enhancement of lung allergic responses. However, unlike the IL-13-dependent enhancement demonstrated for nTreg cells, iTreg cells appeared to mediate increases in lung allergic responses through IL-17, augmenting ongoing type 2-mediated inflammatory responses.

METHODS

Animals

Pathogen-free, 6- to 8-week-old female CD8 α ^{-/-} and IL-13^{-/-} mice were obtained from existing colonies (CD8 α ^{-/-} mice [CD90.2] were provided by Dr Philippa Marrack, as verified by using fluorescence-activated cell signaling (FACS), and IL-13^{-/-} mice were provided by Dr Dale Umettsu, as verified by PCR). C57BL/6 (CD90.1) and IL-17^{-/-} mice were obtained from Jackson Laboratories (Bar Harbor, Me). All mice were maintained on an ovalbumin (OVA)-free diet. All protocols were approved by the Institutional Animal Care and Use Committee at National Jewish Health.

Sensitization and challenge

Sensitization was carried out by means of intraperitoneal injection of 20 μ g of OVA (Sigma-Aldrich, St Louis, Mo) emulsified in 2.25 mg of alum hydroxide (AlumImject; Pierce, Rockford, Ill) in a total volume of 100 μ L on

days 1 and 14. Sensitized and challenged mice (OVA/OVA) and nonsensitized but challenged littermates (PBS/OVA) received aerosol challenges for 20 minutes each day on 3 consecutive days (days 26, 27, and 28) with 1% OVA in PBS by using an ultrasonic nebulizer (Omron, Vernon Hills, Ill).²¹

Measurement of airway responsiveness

Airway responsiveness was assessed, as previously described.²¹ Changes in airway function to increasing concentrations of aerosolized methacholine (MCh) administered for 10 seconds (60 breaths/min, 500- μ L tidal volume) were monitored. Lung resistance (R_L) was continuously computed (Labview; National Instruments, Austin, Tex) by fitting flow, volume, and pressure to an equation of motion. Maximum R_L values were taken and expressed as a percentage change from baseline after PBS aerosol.

Bronchoalveolar lavage

Immediately after AHR measurement, lungs of each animal were lavaged once with 1 mL of 1 $\times$ HBSS from Mediatech (Manassas, Va). Total leukocyte numbers were counted (Countess Automated Cell Counter; Invitrogen, Carlsbad, Calif), and differential cell counts were performed in a blinded manner under light microscopy by counting at least 200 cells on cytocentrifuged preparations (Cytospin 2; Cytospin, Shandon, Runcorn, Cheshire, United Kingdom), stained with Leukostat (Fisher Diagnostics, Middletown, Va), and differentiated by using standard hematologic procedures.

Cell preparation and culture

CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells were enriched from spleens of naive C57BL/6 mice. Lymphocytes were further sorted by means of positive selection for CD4⁺CD25⁺ regulatory T cells with magnetic beads, resulting in a purity of greater than 95% CD4⁺CD25⁺ cells. After depletion of CD4⁺CD25⁺ cells, CD4⁺CD25⁻ T cells in the negative fraction were reisolated by means of positive selection for CD4⁺ T cells to greater than 98% purity with MACS beads (Miltenyi Biotec, Bergisch Gladbach, Germany).

Cells were washed, counted, and resuspended to a final concentration of 4×10^6 cells/mL in complete RPMI 1640 (Mediatech Cellgro, Manassas, Va) tissue culture medium containing heat-inactivated 10% FCS (Sigma-Aldrich), L-glutamine (5 mmol/L), β -mercaptoethanol (2 mmol/L), HEPES buffer (15 mmol/L), penicillin (100 U/mL), and streptomycin (100 μ g/mL; all from Gibco, Grand Island, NY).

In vitro differentiation and adoptive transfer

Isolated CD4⁺CD25⁻ T cells were cultured in X-Vivo 15 (Lonza, Walkersville, Md), supplemented with IL-2 (5 ng/mL), and stimulated with bound anti-CD3 and soluble anti-CD28 (2 μ g/mL) with and without TGF- β (5 ng/mL)²³ and together with or without IL-6 (5 ng/mL) for 5 to 7 days. Differentiated cells were washed and adoptively transferred (5×10^5 in 50 μ L of PBS) into sensitized and nonsensitized recipients before allergen challenge. In some experiments intratracheal instillation of the recombinant protein IL-17 (200 pg) and anti-IL-13, anti-IL-17, anti-GITRL, anti-OX40, and control antibodies (100 μ g) was also performed. Reagents were from eBioscience (San Diego, Calif) and R&D Systems (Minneapolis, Minn).

In some experiments transferred cells (CD90.1⁺) were recovered by using MACS bead sorting after collagenase digestion of lungs from recipient mice (CD90.2⁺) and enriched with nylon wool columns, as previously described.¹²

Measurement of cytokine levels

Cytokine levels in the bronchoalveolar lavage (BAL) fluid and supernatants of *in vitro*-cultured cells were measured by using ELISA: IL-4, IL-5, IL-6, IL-10, IL-13, IL-17, IL-21, IL-22, IFN- γ , and TGF- β from eBioscience. ELISAs were performed according to the manufacturer's directions. Limits of detection were 4 pg/mL for IL-4, IL-5, IL-6, and IL-17; 10 pg/mL for

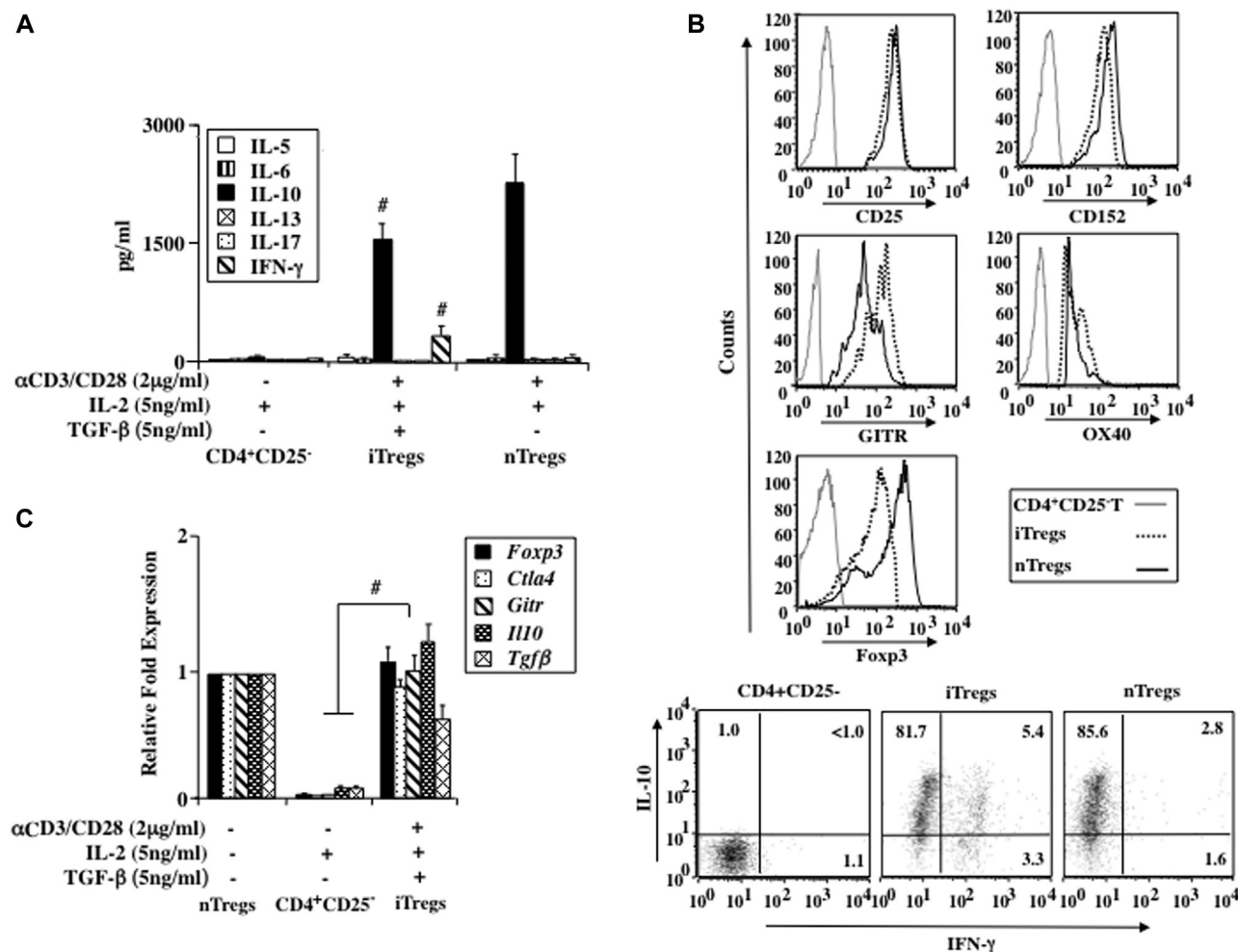


FIG 1. Effect of nTreg cell, iTreg cell, and CD4⁺CD25⁻ T-cell transfer from naive donors into sensitized and challenged CD8^{+/+} recipients. **A**, *In vitro* cytokine production from nTreg cells, iTregs cell, and CD4⁺CD25⁻ T cells. **B**, Flow cytometry of nTreg cells, iTreg cells, and CD4⁺CD25⁻ T cells. **C**, Gene expression levels of nTreg cells, iTreg cells, and CD4⁺CD25⁻ T cells before transfer. #*P* < .05 comparing CD4⁺CD25⁻ T cells with iTreg and nTreg cells. **D-F**, AHR (Fig 1, D), BAL fluid inflammatory cell composition (Fig 1, E), and BAL fluid cytokine levels (Fig 1, F). Shown are means ± SEMs from 3 independent experiments (4 mice/group, *n* = 12). **P* < .01 comparing sensitized and challenged mice (given PBS) with mice challenged alone. †*P* < .05 comparing sensitized and challenged recipients of iTreg and nTreg cells with recipients of PBS or CD4⁺CD25⁻ T cells.

IL-10 and IFN-γ; 8 pg/mL for IL-13 and IL-22; 6 pg/mL for TGF-β; and 16 pg/mL for IL-21.

FACS analysis

Isolated cells, after preincubation with naive mouse serum in staining buffer (PBS, 2% FCS, and 0.2% sodium azide), were labeled with the following conjugated antibodies purchased from BD Biosciences Pharmingen (San Jose, Calif): anti-CD3 fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridinin-chlorophyll-protein complex (PerCP), and allophycocyanin (APC; 17A2); anti-CD4 FITC, PE, PerCP, and APC (L3T4); anti-CD25 FITC (7D4) and PE (PC61); anti-GITR FITC, PE, and APC; and CD152 FITC, PE, and APC. For intracellular staining, cells were stimulated with phorbol 12-myristate 13-acetate (100 ng/mL) and ionomycin (2 μg/mL, Sigma-Aldrich) in complete medium overnight and for 6 hours 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 and APC (JES5-16E3); IL-17 PE and APC (eBio17B7); IFN-γ PE and APC (XMG1.2); TGF-β PE and APC (A75-3.1) Foxp3 PE and APC

(FJK-16s); and RORγt PE and APC (AFKJS-9; eBioscience). Fluorochrome (FITC, PE, PerCP, and APC)-labeled isotype-matched control antibodies were used for background fluorescence staining. Staining was analyzed by using FACScalibur flow cytometry (BD Pharmingen) with CellQuest Pro software. Fluorescence intensity was compared with cells stained with corresponding labeled isotype-matched control antibodies.

Real-time PCR

Total RNA was prepared by using the NucleoSpin RNA II Isolation Kit (Macherey-Nagel, Duren, Germany). cDNA was synthesized with equal amounts of RNA (0.25 μg from recovered cells and 1.0 μg from *in vitro* differentiated cells) by using the QuantiTect Reverse Transcription Kit (Qiagen, Valencia, Calif). Predesigned primers and probes for mouse *Il10* (Mm00439614_m1), *Il13* (Mm00434205_g1), *Il17* (Mm00439618_m1), *Foxp3* (Mm00475162_m1), *RORγt* (Mm01261022_m1), *Tgfb* (Mm01227699_m1), and the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*; Mm99999915_g1) were obtained from Thermo Fisher Scientific (Marietta, Ohio). Real-time PCR (RT-PCR) was performed

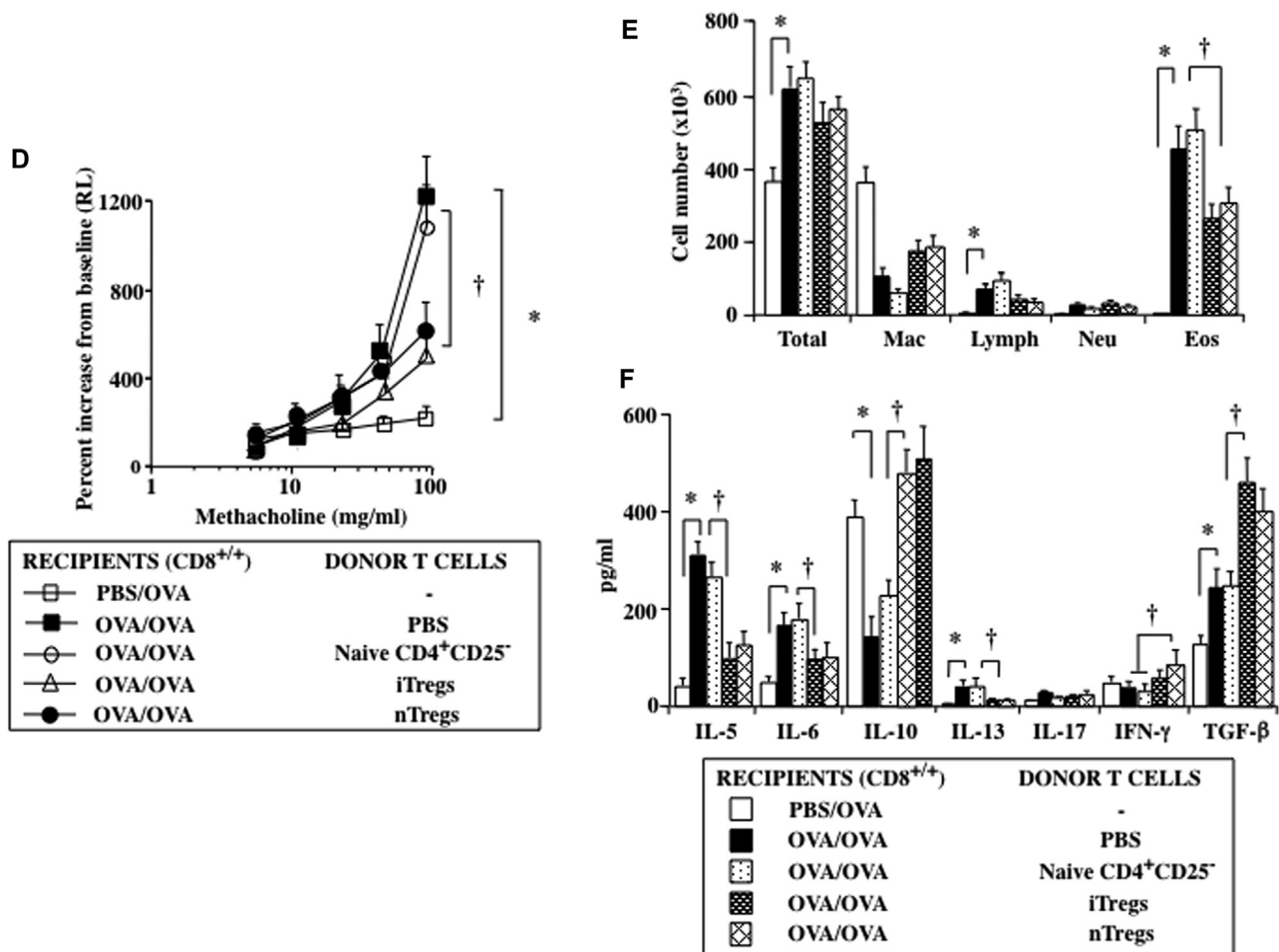


FIG 1. (Continued).

with the ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, Calif). Analysis of relative gene expression was carried out by using the mathematic calculation model based on the $\Delta\Delta$ cycle threshold value, assigning expression levels of nTreg cells and, in some experiments, iTreg cells at 1, as previously described.²⁴

Statistical analysis

ANOVA was used to determine statistical significance. Comparisons for all pairs were performed by using the Tukey-Kramer highest significant difference test. *P* values for significance were set to .05. Values for all measurements were expressed as means $\pm$ SEMs.

RESULTS

Inducible Treg cells regulate development of lung allergic responses in sensitized and challenged WT mice

nTreg cells were shown to be potent suppressors of lung allergic responses in sensitized and challenged WT recipients.^{10,16} The regulatory capacity of *in vitro*-derived iTreg cells was investigated in a similar model of experimental asthma. When compared with nTreg cells, iTreg cells generated from purified naive CD4⁺CD25⁻ T cells in the presence of TGF- β produced significant amounts of IL-10 and moderate amounts of IFN- γ but negligible amounts of IL-5, IL-6, IL-13, and IL-17, as detected by means of ELISA (Fig 1, A) and FACS

analysis (>80% IL-10⁺ iTreg cells; Fig 1, B). Furthermore, similar levels of the transcription factor Foxp3 and CD25, cytotoxic T lymphocyte-associated antigen 4, GITR, and OX40 gene expression by using RT-PCR and protein levels by flow cytometry were expressed in nTreg and iTreg cells (Fig 1, B and C). In contrast, none of these changes were detected in naive CD4⁺CD25⁻ T cells under nonpolarizing (without TGF- β) conditions.

Consistent with previous studies,^{10,16} sensitized and challenged WT mice given PBS or naive CD4⁺CD25⁻ T cells had significant AHR to increasing concentrations of aerosolized MCh, as measured by changes in R_L, eosinophilic inflammation, and increased type 2 cytokine levels in BAL fluid (Fig 1, D-F). Transfer of nTreg or iTreg cells decreased AHR and eosinophilic inflammation. Levels of IL-5, IL-6, and IL-13 were lower in BAL fluid, whereas increased levels of IL-10 and TGF- β were detected. Taken together, the data demonstrated that iTreg and nTreg cells exhibited similar gene and protein profiles and were effective regulators of lung allergic responses when transferred into sensitized and challenged WT recipients.

iTreg cells enhance lung allergic responses in sensitized and challenged CD8^{-/-} mice

Unlike WT mice, CD8^{-/-} mice had limited lung allergic responses after sensitization and challenge (Fig 2, A and B), as

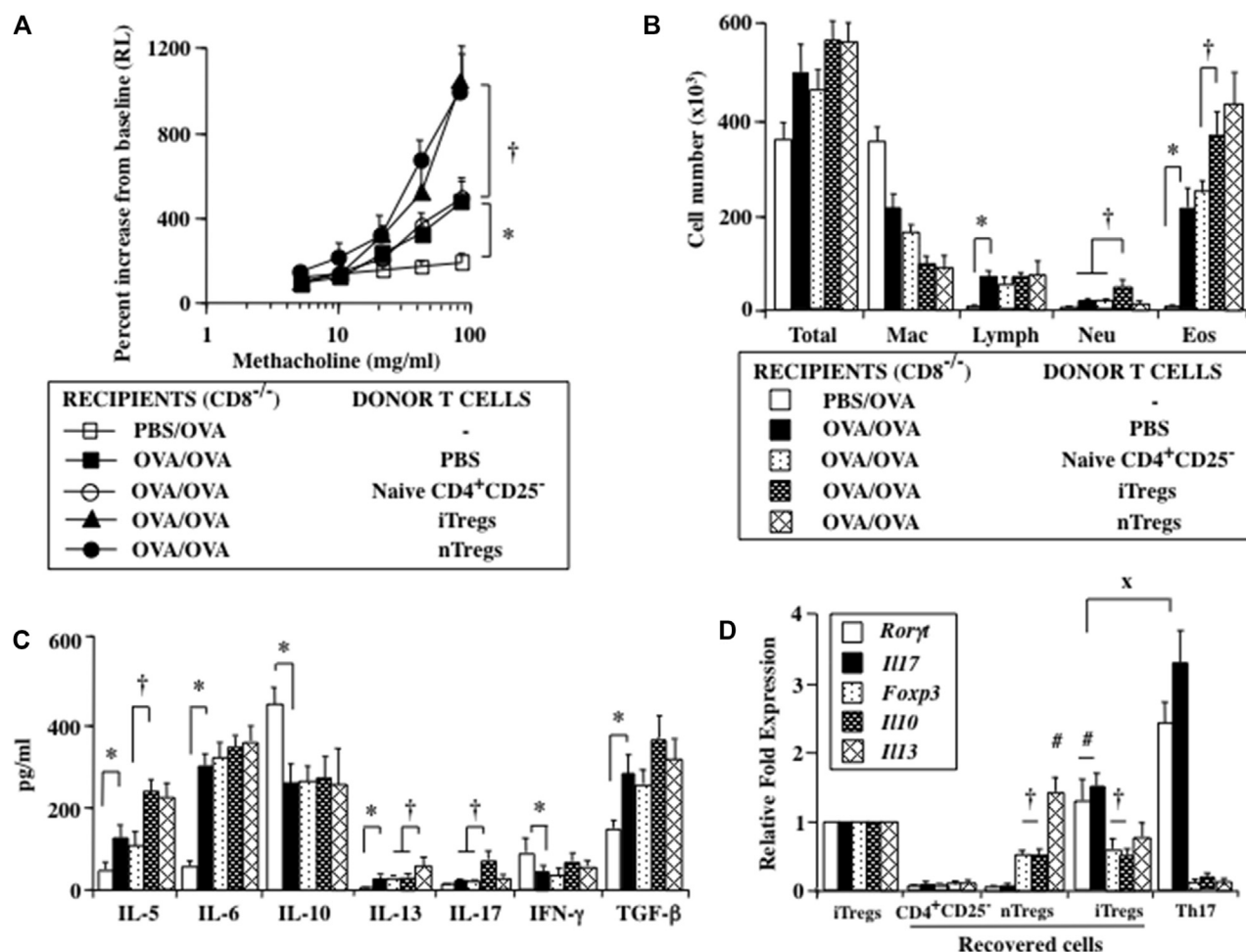


FIG 2. Effect of nTreg cell, iTreg cell, and CD4⁺CD25⁻ T-cell transfer from naive donors into sensitized and challenged CD8^{-/-} recipients. **A-C**, AHR (Fig 2, A), BAL fluid inflammatory cell composition (Fig 2, B), and BAL fluid cytokine levels (Fig 2, C). Shown are means $\pm$ SEMs from 3 independent experiments (4 mice/group, $n = 12$). * $P < .05$ comparing sensitized and challenged mice (given PBS) with mice challenged alone. † $P < .05$ comparing sensitized and challenged recipients of iTreg and nTreg cells with recipients of PBS or CD4⁺CD25⁻ T cells. *Eos*, Eosinophils; *Lymph*, lymphocytes; *Mac*, macrophages; *Neu*, neutrophils. **D**, Gene expression of recovered naive CD4 cells, iTreg cells, Th17 cells, and nTreg cells (compared with levels of pretransferred iTreg cells set at 1). # $P < .05$ (increased) and † $P < .05$ (decreased), X $P < .05$ comparing iTreg cells with Th17 cells.

previously shown.^{6,8-10} In contrast to the suppression of lung allergic responses seen after transfer into sensitized and challenged WT recipients, nTreg cells were shown to enhance lung allergic responses, AHR, and eosinophilic inflammation when transferred into sensitized and challenged CD8^{-/-} recipients (Fig 2, A and B). Associated with these changes were increases in BAL fluid levels of IL-5 and IL-13.

Transfer of iTreg cells into sensitized and challenged CD8^{-/-} recipients, similar to nTreg cells, resulted in significantly increased AHR, beyond levels induced after sensitization and challenge alone (Fig 2, A). Although increases in total cell, lymphocyte, and eosinophil numbers in the BAL fluid were detected in sensitized and challenged CD8^{-/-} mice compared with those challenged only, further increases in eosinophil and neutrophil numbers were observed in recipients of iTreg cells (Fig 2, B). This was associated with increased IL-5 and IL-17 levels in the BAL fluid of sensitized and challenged CD8^{-/-} recipients; unlike transfer of nTreg cells, IL-13 levels were not increased after transfer of iTreg cells (Fig 2, C).

To further elucidate the molecular basis for the conversion of transferred cells, expression of *Foxp3*, *Il10*, *Tgfb*, *RORγt*, *Il13*, and *Il17* in transferred and recovered nTreg and iTreg cells from the lungs of sensitized and challenged recipients were analyzed by using RT-PCR; gene expression levels were normalized to levels seen in iTreg cells before transfer (taken as baseline and assigned a value of 1). Transferred nTreg and iTreg cells (CD90.1⁺) were recovered from the lungs of CD90.2⁺ recipient mice, which comprised more than 98% of the cells after selection. In parallel to the changes in lung physiology and inflammation, similar to findings in transferred and recovered nTreg cells, significant decreases in gene expression of *Foxp3* and *Il10* were detectable in iTreg cells. nTreg cells expressed negligible levels of *RORγt* and *Il17* (Fig 2, D), whereas iTreg cells recovered from the lungs exhibited increased *RORγt* and *Il17* expression (Fig 2, D). Unlike nTreg cells, which expressed high levels of *Il13*, little gene expression was detected in the iTreg cells. When compared with *in vitro*-differentiated Th17 cells, expression levels of *RORγt* and *Il17* in recovered iTreg cells

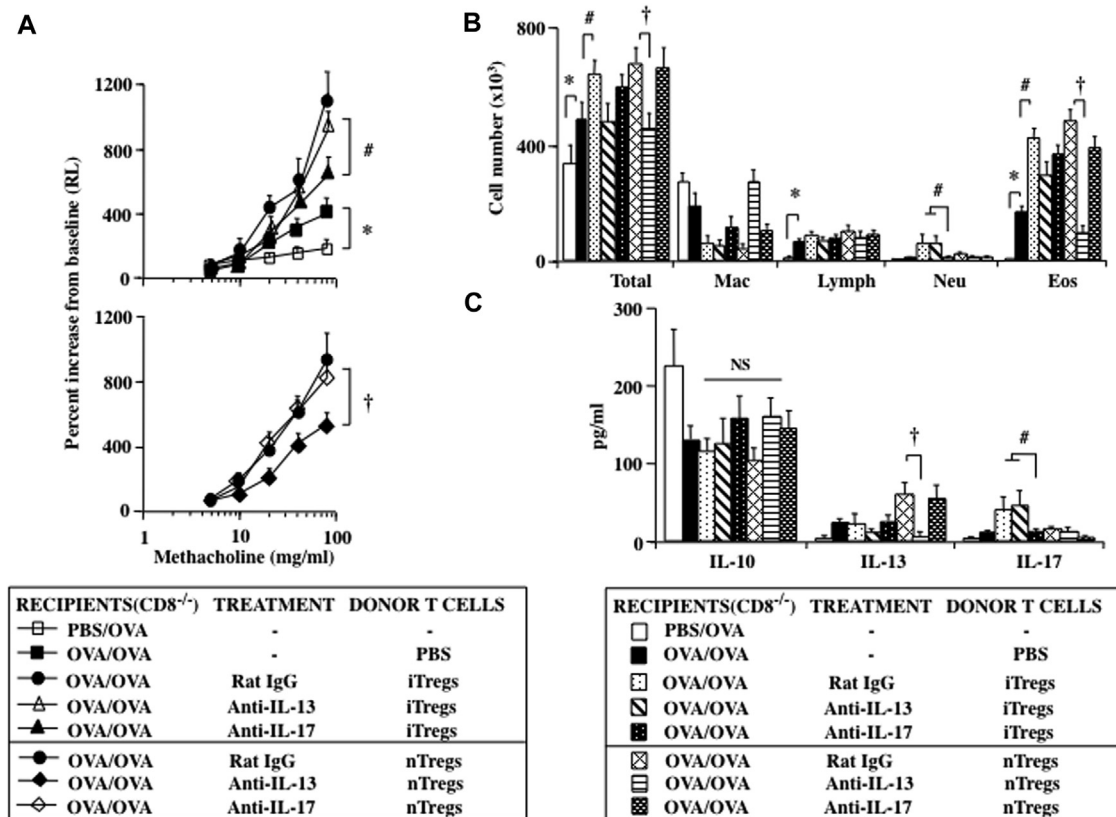


FIG 3. Effect of administering anti-IL-13 or anti-IL-17 antibody on enhancement of lung allergic responses in sensitized and challenged CD8^{-/-} recipients of iTreg and nTreg cells. AHR (A), BAL fluid inflammatory cell composition (B), and BAL fluid cytokine levels (C) are shown. Shown are means $\pm$ SEMs from 3 independent experiments (4 mice/group, $n = 12$). *Eos*, Eosinophils; *Lymph*, lymphocytes; *Mac*, macrophages; *Neu*, neutrophils. * $P < .05$ comparing sensitized and challenged mice (given PBS) with mice challenged alone. # $P < .05$ comparing recipients of iTreg cells given control and anti-IL-13 antibody with recipients given anti-IL-17. † $P < .05$ comparing recipients of nTreg cells given control and anti-IL-17 antibody with recipients given anti-IL-13 antibody. NS, Not significant.

were lower, and mRNA levels of *Foxp3* and *Il10* remained higher. These data demonstrated that TGF- β -induced *Foxp3*⁺ iTreg cell-mediated enhancement of lung allergic responses after transfer into sensitized and challenged CD8^{-/-} recipients was associated with increased levels of IL-17 gene and protein expression, unlike the findings in nTreg cells.

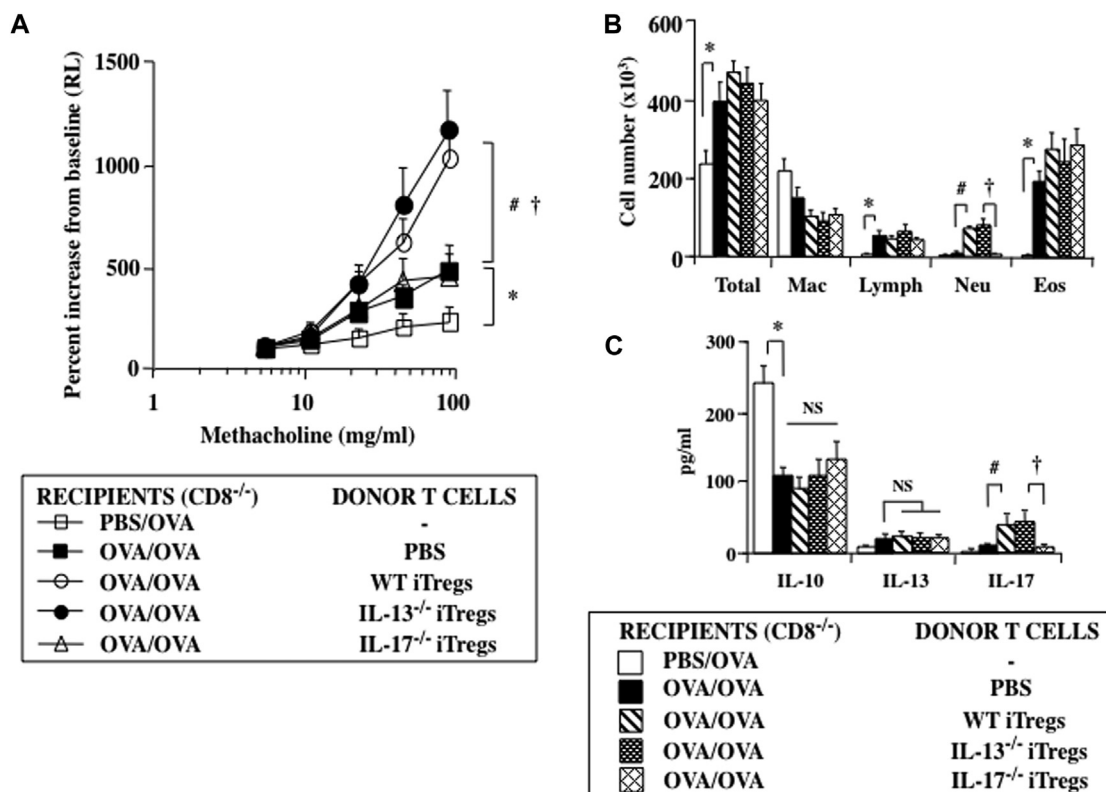
Anti-IL-17, but not anti-IL-13, prevents iTreg cell-mediated enhancement of lung allergic responses

To determine whether IL-17 was essential to the iTreg cell-mediated enhancement of lung allergic responses in CD8^{-/-} recipients, anti-IL-13 or anti-IL-17 was administered to sensitized CD8^{-/-} recipients just before intratracheal transfer of iTreg or nTreg cells and allergen challenge. In contrast to recipients of iTreg cells treated with control antibody, administration of anti-IL-17, but not anti-IL-13, attenuated enhancement after iTreg cell transfer, with significant reductions in AHR and airway neutrophilia but little change in airway eosinophilia (Fig 3). Paralleling the changes in lung physiology, administration of anti-IL-17 resulted in a significant reduction in the amount of IL-17 detected in the BAL fluid with little change in IL-13 levels (Fig 3, C). Conversely, treatment with anti-IL-13

prevented the enhancement mediated by nTreg cells in association with decreased BAL levels of IL-13 and airway eosinophilia compared with responses in recipients of nTreg cells treated with control antibody. Neither treatment significantly altered IL-4 or IL-5 levels (data not shown). These data demonstrated that events associated with the functional plasticity and pathogenic conversion of iTreg and nTreg cells for the enhancement of lung allergic responses were clearly distinguishable: iTreg cells converting into T_H17 cells producing IL-17 and nTreg cells becoming type 2 effector cells releasing IL-13.

IL-13^{-/-} but not IL-17^{-/-} iTreg cells enhance lung allergic responses in sensitized and challenged CD8^{-/-} mice

The critical role of IL-17 but not IL-13 in the iTreg cell-mediated enhancement of lung allergic responses in sensitized and challenged CD8^{-/-} mice was further demonstrated by transferring iTreg cells generated from naive CD4⁺CD25⁻ T cells isolated from IL-13^{-/-} and IL-17^{-/-} mice. In contrast to iTreg cells from WT or IL-13^{-/-} mice, iTreg cells from IL-17^{-/-} mice did not increase AHR or airway neutrophilia in sensitized and challenged CD8^{-/-} recipients. Eosinophil numbers in these recipients were unchanged, as



were IL-10 and IL-13 levels; IL-17 levels in BAL fluid were significantly lower compared with those in recipients of WT and IL-13^{-/-} iTreg cells (Fig 4).

T_H17 cells or exogenous IL-17 enhance lung allergic responses in sensitized and challenged CD8^{-/-} recipients

A role for T_H17 cells and airway neutrophilia in asthmatic patients has been reported in human subjects and mice, although the results were somewhat inconsistent.^{25,26} An association with a more severe asthma phenotype that responds poorly to treatment with corticosteroids has also been reported.²⁷ The combination of the polarizing cytokines IL-6 and TGF-β was critical for *in vitro* and *in vivo* generation of T_H17 cells^{24,28,29} and conversion of Treg cells into T_H17 cells.^{30,31} Although the evidence suggested that transferred iTreg cells had the capacity to acquire a T_H17 phenotype expressing the transcription factor *RORγt* and became IL-17-producing cells in the lungs of recipient mice, it was not clear whether the enhancement of lung allergic responses was dependent solely on T_H17 cells and IL-17. To this end, the effect of administering *in vitro*-generated T_H17 cells and exogenous IL-17 protein was assessed in sensitized and challenged and challenged-only CD8^{-/-} mice. Purified naive CD4⁺CD25⁻ T cells were differentiated into T_H17 cells under

polarizing conditions with IL-6 and TGF-β.^{28,29,31} In contrast to cells stimulated with TGF-β alone, increased levels of IL-17, IL-21, and IL-22 were detected in supernatants of cells cultured together with IL-6 (Fig 5, A).

Intratracheal administration of T_H17 cells or IL-17 protein into sensitized and challenged CD8^{-/-} recipients resulted in the enhancement of AHR, which was similar to findings observed after transfer of iTreg cells (Fig 5, B). The enhancement of AHR seen with T_H17 cells or IL-17 was associated with increased airway neutrophilia and BAL IL-17 levels; airway eosinophilia was unaltered (Fig 5, C and D). However, T_H17 cells or IL-17 alone in the absence of sensitization were insufficient to induce significant AHR, eosinophilic, and neutrophilic inflammation or alter cytokine levels (other than IL-17) in CD8^{-/-} recipients. These data imply that the immune status and local inflammatory environment in recipient mice contributed to the enhancement of lung allergic responses. In the absence of ongoing lung inflammation, T_H17 cells or IL-17 had little effect on these changes.

Anti-IL-6, but not anti-TGF-β, anti-GITR, or anti-OX40, prevents conversion of iTreg cells in sensitized and challenged CD8^{-/-} mice

Because iTreg cells in the absence of exogenous IL-6 did not produce IL-17 *in vitro* (Fig 6, A), detection of IL-17 in BAL fluid

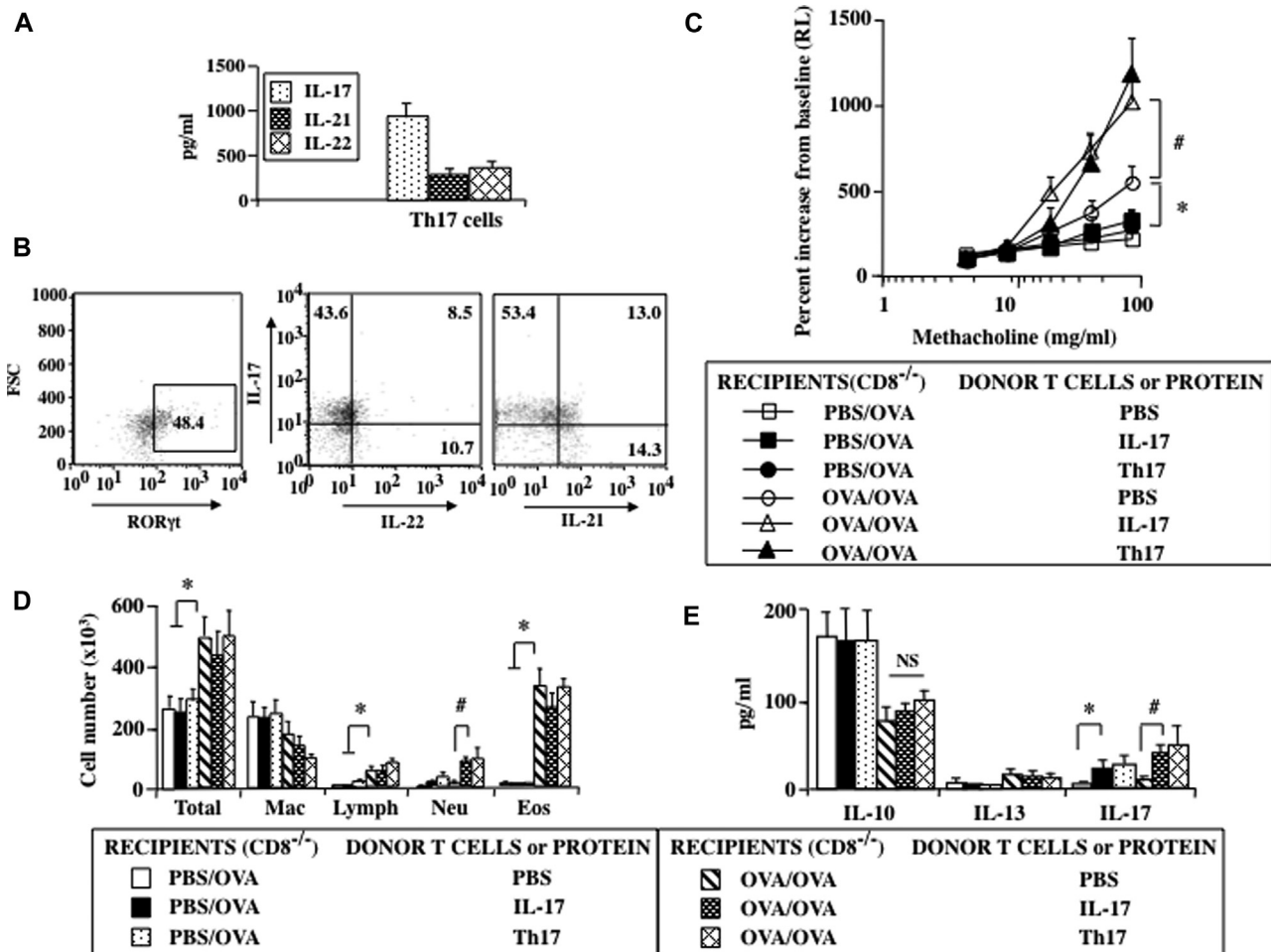


FIG 5. Effect of administering T_H17 cells or IL-17 on lung allergic responses in sensitized and challenged and challenged only $CD8^{-/-}$ recipients. Cytokine production (**A**) and FACS analysis (**B**) of T_H17 cells, AHR (**C**), BAL fluid cell composition (**D**), and BAL fluid cytokine levels (**E**) are shown. Results shown are means $\pm$ SEMs from 3 independent experiments ($n = 12$). *Eos*, Eosinophils; *Lymph*, lymphocytes; *Mac*, macrophages; *Neu*, neutrophils. * $P < .05$ comparing sensitized and challenged mice (given PBS) with mice challenged alone given PBS, IL-17, or T_H17 cells. # $P < .05$ comparing sensitized and challenged recipients of IL-17 protein and T_H17 cells to recipients of PBS. NS, Not significant.

coupled with enhancement of lung allergic responses suggested that recipient mice might be the source of IL-6. Furthermore, despite expression of the surface molecules GITR and OX40, production of IL-17, IL-21, and IL-22 from iTreg cells detected after addition of IL-6 and T_H17 cells was not influenced by ligation of GITR or OX40, unlike alterations seen with nTreg cells exposed to these proteins in previous studies.⁸⁻¹⁰

Increased levels of IL-6 after sensitization and challenge were reported in both WT and $CD8^{-/-}$ mice.^{10,18,24} The role of host (recipient) IL-6 and TGF- β in the *in vivo* development of iTreg cells into IL-17⁺ T effector cells was assessed by administering anti-IL-6 or anti-TGF- β to recipients before iTreg cell transfer. The effects of anti-GITR and anti-OX40 on iTreg cells *in vivo* was also investigated. In recipients of iTreg cells, administration of anti-IL-6, but not anti-TGF- β , anti-GITR, or anti-OX40, prevented enhancement of lung allergic responses and airway neutrophilia in association with lower levels of IL-17 in the BAL fluid (Fig 6, B-D). Because only IL-6 appeared critical to enhancement, transferred CD90.1⁺ cells were recovered from the lungs of CD90.2⁺ recipients for flow cytometric analysis of

intracellular IL-17 and RORγt. Fewer cells from anti-IL-6-treated mice expressed IL-17 and RORγt compared with those from mice that received control antibody (Fig 6, E). These findings confirmed that host-derived IL-6 was essential to the pathogenic conversion of iTreg cells to IL-17 production in the airways of recipient mice.

DISCUSSION

Constitutive expression of the lineage-specific “master” transcription factor Foxp3 in $CD4^+CD25^+$ nTreg cells confers a set of unique surface markers, development-related anergy, and capacity to produce important immunoregulatory cytokines, such as IL-10 and TGF- β .³ The critical role of nTreg cells in the maintenance of immunologic tolerance and prevention of autoimmunity is highlighted in human X-linked immune dysregulation, polyendocrinopathy, and enteropathy disease and in the Scurfy mouse.^{1,2} As observed in many T-cell subsets, the ability of nTreg cells to exhibit a suppressive phenotype does not appear terminally fixed, so that under different environmental

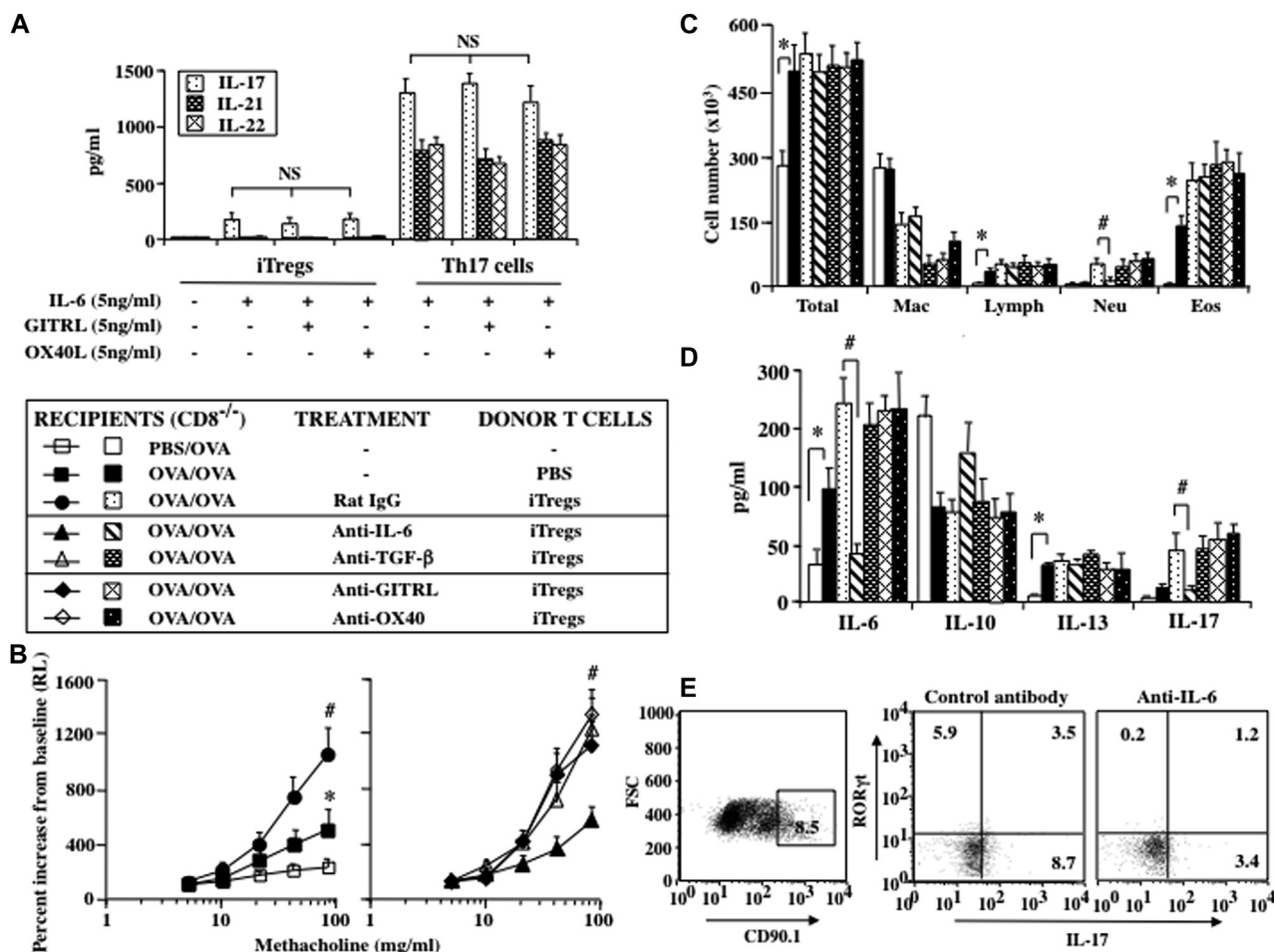


FIG 6. Effect of administering control IgG, anti-IL-6, anti-TGF- β , anti-GITRL, or anti-OX40 antibody on lung allergic responses in sensitized and challenged CD8^{-/-} recipients of iTreg cells. **A**, Effect of GITRL and OX40 ligand on *in vitro* cytokine production from iTreg cells and Th17 cells. **B-E**, AHR (Fig 6, B), BAL fluid inflammatory cell composition (Fig 6, C), BAL fluid cytokine levels (Fig 6, D), and flow cytometry of recovered iTreg cells (Fig 6, E). Shown are the means $\pm$ SEMs from 3 independent experiments (4 mice/group, $n = 12$). * $P < .05$ comparing sensitized and challenged mice (given PBS) with mice challenged alone. # $P < .05$ comparing recipients of iTreg cells given control and anti-TGF- β , anti-GITRL, and anti-OX40 antibody with recipients given anti-IL-6. NS, Not significant.

conditions, phenotypic and functional plasticity was demonstrated with changes in gene expression, surface receptor expression, and cytokine production.^{6,8} As a result, the *in vivo* functional activity of nTreg cells was altered from suppression of lung allergic responses when transferred into sensitized and challenged WT recipients compared with enhancement of these responses after transfer into CD8^{-/-} recipients. Use of CD8^{-/-} mice to reveal the functional conversion and transcriptional reprogramming of the nTreg cells was in keeping with data demonstrating the critical need for CD8–nTreg cell interactions to maintain the suppressive phenotype. Loss of the suppressive phenotype was observed when the CD8 signal was eliminated and nTreg cells became producers of IL-6.¹⁸ Enhancement of lung allergic responses was shown to be dependent on the production of IL-13 by the nTreg cells themselves.⁸⁻¹⁰ In the present study we compared the events associated with nTreg cell conversion with those of iTreg cells under similar conditions. The data identified comparable suppressive activity in both cell types when transferred into WT recipients and conversion to an

enhancing phenotype in CD8^{-/-} recipients, but these outcomes were mediated through different pathways.

An experimental model of asthma with MCh-induced AHR, airway eosinophilia, and type 2 cytokine production was used to monitor the *in vivo* activities of nTreg and iTreg cells after transfer into sensitized and challenged WT and CD8^{-/-} recipients. Similar to other studies,^{4,7,11} iTreg cells were effective regulators of these lung allergic responses, suppressing the development of AHR and eosinophilic inflammation in WT recipients when transferred after sensitization and before allergen challenge. Overall, the suppressive activity exhibited by iTreg cells was comparable with that seen after transfer of nTreg cells.^{6,12,16} In both cases IL-5, IL-6, and IL-13 levels in BAL fluid were reduced, whereas IL-10, IFN- γ , and TGF- β levels were increased. Whereas Foxp3 expression was constitutive in CD4⁺CD25⁺ nTreg cells, T-cell receptor stimulation of CD4⁺CD25⁺ T cells alone was insufficient and required addition of TGF- β for induction of Foxp3⁺ iTreg cells, as shown in a dose-dependent⁴ and time-dependent⁵ manner.

Given the similarities in functional outcomes (suppression) in WT recipients, we determined the consequences of transfer into CD8^{-/-} recipients. Compared with WT mice, CD8^{-/-} mice had limited lung allergic responses after sensitization and challenge with allergen.^{7,14,18} Recipients of iTreg cells had additional increases in AHR and airway eosinophilia similar to the enhancement seen in recipients of nTreg cells. Unlike recipients of nTreg cells, recipients of iTreg cells also had significant airway neutrophilia in association with increased IL-17 levels in the BAL fluid. Because little IL-17 gene or protein expression was detected in iTreg cells before transfer and increased levels of IL-17 in BAL fluid were only observed in recipients of iTreg cells, this suggested *in vivo* transdifferentiation and conversion of the transferred iTreg cells. This was supported by findings in transferred iTreg cells, which were recovered from the lungs of sensitized and challenged CD8^{-/-} recipients by using specific markers; in the recovered iTreg cells reduced *Foxp3* and *Il10* mRNA levels but higher mRNA and protein levels of ROR γ t and IL-17 were demonstrated. These findings were in contrast to transferred and recovered nTreg cells in which lower gene expression levels of *Foxp3* and *Il10* and higher gene expression levels of *Il13* were shown here and in previous studies.^{8-10,18} Thus it appears that the differentiation of nTreg and iTreg cells from a shared Foxp3⁺ suppressor phenotype (in CD8^{+/-} recipients) to divergent pathogenic effector lineages characterized by IL-13⁺ and IL-17⁺ effector T cells in CD8^{-/-} recipients resulted from the absence of CD8 signaling.

The requirements for IL-6, TGF- β , and the lineage-specific transcription factor ROR γ t are well established for the *in vitro* differentiation of T_H17 cells.^{24,28} The *in vivo* conversion of iTreg cells from suppressors to IL-17⁺ effector T cells in the lungs of sensitized and challenged CD8^{-/-} recipients suggested that host (recipient)-derived IL-6 was critical. This notion was confirmed by preventing enhancement of lung allergic responses in recipients treated with anti-IL-6 antibody before adoptive transfer. The importance of IL-6 in the *in vitro* and *in vivo* differentiation of T_H17 cells was not surprising given the pleiotropic activities of this cytokine in influencing inflammatory responses. IL-6 signaling induces phosphorylation of signal transducer and activator of transcription 3, which is required for inducing ROR γ t,³¹ promoting T_H17 cell differentiation,^{24,30} reducing TGF- β -induced expression of Foxp3,²⁹ and abrogating suppression of nTreg cells in association with decreased expression of Foxp3.^{18,21}

Conversion of CD4⁺CD25⁻ T cells into iTreg cells was shown to critically depend on TGF- β .^{4,5} Once differentiated to iTreg cells with TGF- β and transferred into sensitized and challenged recipients, enhancement of lung allergic responses was no longer dependent on TGF- β because treatment of recipients with anti-TGF- β did not prevent iTreg cell-mediated enhancement. In contrast, treatment of the recipients with anti-IL-6 prevented enhancement and T_H17 differentiation after iTreg cell transfer. Although TGF- β contributed to the differentiation programming of both iTreg and T_H17 cells, it appeared that *in vivo* IL-6 from the hosts favored T_H17 development and IL-17 release at the expense of iTreg cell development, maintenance, or both^{24,28-30} and, as shown here, resulted in enhancement (and not suppression) of lung allergic responses in sensitized and challenged recipients. These findings suggested that iTreg cell conversion to a T_H17-like cell proceeded in a staged manner; the initial induction events for iTreg cell differentiation were dependent on TGF- β , but later

events were TGF- β independent and required IL-6. However, IL-6 alone was not sufficient because in the absence of TGF- β , IL-6 stimulation did not induce the development of T_H17 cells.^{24,28}

Differences in transcriptional reprogramming of nTreg and iTreg cells were evident in the terminal events, resulting in enhancement responses. Enhancement of lung allergic responses mediated by nTreg cells was attenuated by treatment of recipients with anti-IL-13, whereas the same antibody had no effect on the events mediated by transferred iTreg cells. Reciprocally, anti-IL-17 had no effect on the events triggered by transferred nTreg cells but markedly reduced the outcomes mediated by transferred iTreg cells. The critical requirement for IL-17 in the enhancement of lung allergic responses was also demonstrated by the absence of such increases in recipients of IL-17-deficient iTreg cells. Whereas the role of IL-13 is firmly established in experimental models of asthma and in asthmatic patients,³²⁻³⁴ the contribution of T_H17 cells and IL-17 remains somewhat controversial. Although T_H17-dependent neutrophilia in the airways has been observed,^{25-27,35} the role of neutrophils in development of altered airway function is not entirely clear. Inhibition of early airway neutrophilia had little effect on later development of AHR,³⁶ and blocking the activity of a neutrophil chemoattractant had little benefit in asthmatic patients.³⁷ In allergen challenge models in mice, the accumulation of neutrophils appeared early and transiently and was later followed by the influx of eosinophils.³⁸ After intratracheal administration of T_H17 cells or IL-17 protein into sensitized and challenged CD8^{-/-} recipients, AHR and airway inflammation, including airway neutrophilia, were increased. Similar to findings after transfer of iTreg cells, enhancement seen with T_H17 cells or IL-17 was associated with increased airway neutrophilia and increased BAL IL-17 levels. However, T_H17 cells or IL-17 alone were insufficient to induce any significant AHR or eosinophilic or neutrophilic inflammation or alter cytokine levels (other than IL-17) in nonsensitized CD8^{-/-} recipients. Thus IL-17, neutrophils, or both likely play the role of “amplifiers,” augmenting an established allergic immune response.

There is now compelling evidence demonstrating that Foxp3⁺ Treg cells have the potential to undergo functional reprogramming to acquire an effector/helper phenotype. The plasticity of nTreg and iTreg cells was influenced by extracellular stimuli, intracellular signaling molecules, or both that are associated with alterations in levels of Foxp3 expression. It is likely that a complex integration of different signals dictates the final profile of subsets of regulatory T cells in the lungs or other tissues. Our previous findings in nTreg cells suggested that these signals might be hierarchical in that signals mediated by CD8–nTreg cell interactions superseded those mediated through ligation of GITR, abrogating enhancement and maintaining suppressor activity.¹⁰ Inhibition or interference with the interaction/engagement of MHC class I on nTreg cells with CD8 effectively abrogated suppression.⁶ In addition, interaction of CD8 and MHC class I was demonstrated to be critical in the maintenance of suppressive activities in the periphery by controlling endogenous expression of IL-6.¹⁸ Thus an alteration in Treg cell function has been reported after ligation of GITR⁸⁻¹⁰ and OX40³⁹ or through IL-6–IL-6 receptor interactions.^{18,19,21} In nTreg cells GITR stimulation was shown to abrogate suppressive activity and blockade of signaling through GITR effectively prevented both the loss of the

suppressor phenotype and conversion to pathogenic IL-13⁺CD4⁺ T effectors. Despite expression of these receptors on both nTreg and iTreg cells, signaling through neither GITR nor OX40 appeared to influence the *in vitro* and *in vivo* conversion of iTreg cells to IL-17⁺CD4⁺ effector T cells and production of IL-17. Similarly, stimulation of T_H17 cells with GITRL or OX40 ligand did not alter IL-17 production.

In summary, the data demonstrated an important balance in the lung between iTreg cells and Th17 cells after sensitization and challenge, emphasizing that the functional outcomes of nTreg and iTreg cells in the lung were dependent on cues in the host environment. The findings further extend the role of the proinflammatory cytokine IL-6 in overriding a dominant program of Treg cell function in the lung to favor T_H17 differentiation. In WT (CD8^{+/+}) recipients both nTreg and iTreg cells were effective suppressors of the development of lung allergic responses, whereas in the absence of host CD8 signaling, these same cells acquired specific effector functions, enhancing lung allergic responses as a consequence of divergent pathways. The reprogramming pathways and enhancement appeared to be distinct and cytokine specific in that IL-13 production was GITR dependent in nTreg cells, whereas in iTreg cells IL-17 production was GITR independent but IL-6 dependent. The IL-13-producing “ex-nTreg cells” mediated an eosinophil-predominant response in the lung, whereas the IL-17-producing “ex-iTreg cells” elicited a neutrophil-associated enhancement. Despite these final effector differences, the fate of nTreg and iTreg cells after transfer shared many features, including a reduction in Foxp3 expression, loss of a specific regulatory phenotype and function, and the gain of distinct effector T-cell functions. The ability to manipulate regulatory T-cell function through biological manipulation has clinical implications for the treatment of immune/inflammatory disorders.

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Clinical implications: Regulatory T cells are suppressors of the development of allergic asthma but are capable of converting into pathogenic effector cells. Maintaining suppressive activity might be essential in controlling immune/inflammatory responses.

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