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

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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, *RORγt* 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 2017;139:1331-42.)

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

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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 syndrome[1] 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-specific[14] 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

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 demonstrated[17] and shown to be necessary for expression of suppressor activity[6] 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 $CD8\alpha^{-/-}$ and $IL\text{-}13^{-/-}$ mice were obtained from existing colonies ($CD8\alpha^{-/-}$ mice [CD90.2] were provided by Dr Philippa Marrack, as verified by using fluorescence-activated cell signaling (FACS), and $IL\text{-}13^{-/-}$ mice were provided by Dr Dale Utmetsu, as verified by PCR). C57BL/6 (CD90.1) and $IL\text{-}17^{-/-}$ 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 μg 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 μL 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-μL 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× 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 μg/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 μg/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 μL 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 μg) 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

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