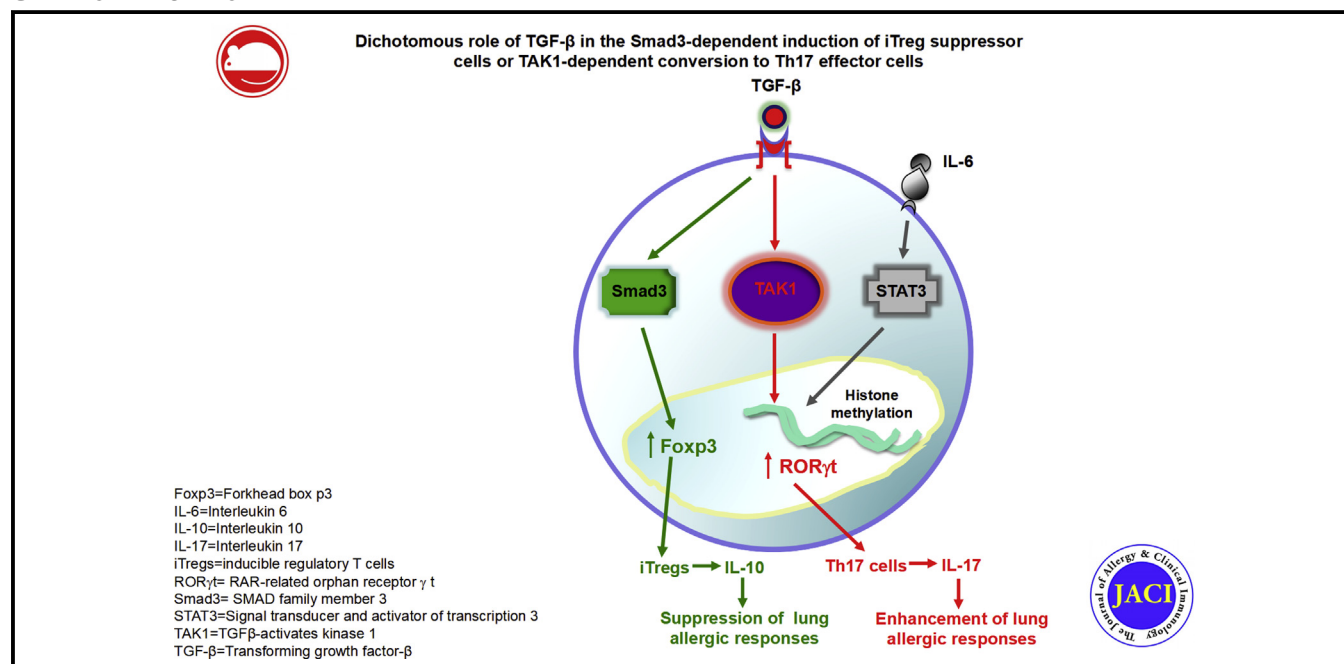


Dichotomous role of TGF- β controls inducible regulatory T-cell fate in allergic airway disease through Smad3 and TGF- β -activated kinase 1



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GRAPHICAL ABSTRACT



Background: Inducible CD4⁺CD25⁺ regulatory T (iTreg) cells can become pathogenic effector cells, enhancing lung allergic responses.

Objective: We aimed to define the underlying cellular and molecular pathways activated by TGF- β , which determine the suppressor or enhancing activities of iTreg cells.

Methods: Sensitized wild-type and CD8-deficient (CD8^{-/-}) mice were challenged with allergen. Isolated CD4⁺CD25⁺ T cells were activated by using anti-CD3/anti-CD28. To generate suppressor iTreg cells, cells were then differentiated in the presence of TGF- β , whereas IL-17-producing effector T cells were additionally exposed to IL-6. After TGF- β , Smad3 and TGF- β -activated kinase 1 (TAK1) kinase levels were

monitored. The consequences of inhibiting either kinase were determined *in vitro* and after transfer into CD8^{-/-} recipients. Quantitative PCR and chromatin immunoprecipitation were used to monitor gene expression and histone modifications at the retinoic acid-related orphan receptor γ t (*Ror γ t*) locus.

Results: In wild-type mice, iTreg cells suppressed lung allergic responses linked to Smad3-dependent forkhead box P3 (Foxp3) expression and IL-10 production. In the presence of IL-6, iTreg cells converted to T_H17 cells, mediating a neutrophil-dependent enhancement of lung allergic responses in CD8^{-/-} mice.

Conversion was regulated by TAK1. Inhibition or silencing of TAK1 prevented expression of *Ror γ t* and T_H17 differentiation through histone modifications of *Ror γ t*; Foxp3 expression and

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iTreg cell-mediated suppression remained intact. In the same cell, TGF- β induced coexpression of Smad3 and TAK1 proteins; in the presence of IL-6, expression of Smad3 and Foxp3 but not TAK1 decreased.

Conclusion: TGF- β regulates iTreg cell outcomes through 2 distinct signal transduction pathways: one Smad3 dependent and the other TAK1 dependent. The balance of these pathways has important implications in T_H17-mediated autoimmune diseases and neutrophil-dependent asthma. (J Allergy Clin Immunol 2020;145:933-46.)

Key words: Inducible CD4⁺CD25⁺ regulatory T cells, T_H17 cells, epigenetics, allergic lung, Smad3, forkhead box P3, TGF- β -activated kinase 1, retinoic acid-related orphan receptor γ t, IL-17

Inducible CD4⁺CD25⁺ regulatory T (iTreg) cells comprise a subset of forkhead box P3 (Foxp3)⁺CD4⁺ suppressor T cells, which together with naturally occurring CD4⁺CD25⁺ regulatory T (nTreg) cells are critical for immune homeostasis and prevention of autoimmunity.¹ In contrast to thymus-derived nTreg cells, development of iTreg cells occurs in peripheral lymphoid tissues, generally in inflammatory or tolerogenic settings.²⁻⁴ *In vitro* iTreg cells are generated by culturing activated T cells (anti-CD3/CD28) with TGF- β .⁵ The functional fidelity and stability of Treg cells are not fixed, but these cells can undergo transcriptional reprogramming in response to environmental cues, resulting in decreased expression of the transcription factor Foxp3, loss of suppressor activity, and gain of effector T (Teff) cell function.⁵⁻⁹ In addition to the inflammatory environment, host immune status, including the presence or absence of CD8⁺ T cells, appears to be critical in determining the functional outcome and cell fate of Treg cells.^{5,6,10,11}

Inducible Treg cells have been shown to be important and effective regulators of lung allergic responses in sensitized and challenged wild-type (WT; CD8^{+/+}) recipients.^{5,12,13} In contrast to the suppression seen when transferred into WT recipients, both nTreg and iTreg cells exhibited *de novo* effector function, resulting in enhancement of lung allergic responses when transferred into CD8-deficient (CD8^{-/-}) mice generated by means of gene knockout or after antibody depletion.^{5,10,11} In the absence of CD8 interaction/engagement with the MHC complex I of Treg cells, suppressor function was abrogated, and conversion of Treg to Teff cells was promoted by different factors and signaling pathways, resulting in enhancement of lung allergic responses.^{5,6,10,11} Conversion signals were overridden after reconstitution of CD8⁺ T cells in CD8^{-/-} recipient mice, suggesting the hierarchical role of CD8-MHC complex I signaling in regulating cell fate and preventing Treg cell-mediated enhancement of lung allergic responses.¹⁴ Hence experimental models of asthma in WT (CD8^{+/+}) and CD8^{-/-} mice have served as valuable models to assess the role and balance of different signal transduction pathways in dictating the plasticity and functional outcomes of Treg cells, suppressor function, or acquisition of effector function.^{5,6,10,11,14}

Despite a shared outcome, the pathways underlying enhancement and the associated composition of lung inflammatory cells were distinct in these 2 regulatory subsets. Eosinophil-dominant enhancement by nTreg cells was dependent on activation of c-Jun

Abbreviations used

AHR:	Airway hyperresponsiveness
APC:	Allophycocyanin
BAL:	Bronchoalveolar lavage
CNS:	Conserved noncoding sequence
FITC:	Fluorescein isothiocyanate
Foxp3:	Forkhead box P3
iTreg:	Inducible CD4 ⁺ CD25 ⁺ regulatory T
nTreg:	Naturally occurring CD4 ⁺ CD25 ⁺ regulatory T
OVA:	Ovalbumin
PE:	Phycoerythrin
PerCP:	Peridinin-chlorophyll-protein complex
ROR γ t:	Retinoic acid-related orphan receptor γ t
shRNA:	Short hairpin RNA
TAK1:	TGF- β -activated kinase 1
TCR:	T-cell receptor
Teff:	Effector T
WT:	Wild-type

N-terminal kinase 2 through ligation of glucocorticoid-induced TNF receptor-related protein, resulting in conversion of nTreg cells into IL-13-producing Teff cells.^{5,14,15} In contrast, *in vitro* and *in vivo* conversion of iTreg cells into IL-17-producing Teff cells, resulting in neutrophil-mediated enhancement and expression of the lineage-specific transcription factor retinoic acid-related orphan receptor γ t (ROR γ t), was critically dependent on IL-6.^{5,16-18}

In vitro development of iTreg cells from naive CD4⁺CD25⁻ T cells and transcription of *Foxp3* was dependent on signals through both the T-cell receptor (TCR) and TGF- β .^{5,19,20} Mice with a deficiency of TGF- β similar to mice lacking Foxp3²¹ had a fatal autoimmune syndrome associated with defects in Treg cell function and/or numbers in the periphery.²² Paradoxically, TGF- β was also required for T_H17 commitment. Its overexpression resulted in enhanced IL-17 production,¹⁶ and conversely, deficiency caused by gene knockout¹⁸ or interference of TGF- β signaling in T cells²³ attenuated T_H17-mediated disease. Although signaling by TGF- β through Smad3 was essential to the expression of Foxp3²⁰ and development of iTreg cells,^{24,25} the role of TGF- β signaling in the development of T_H17 cells and ROR γ t expression is less well defined.

The present study identifies the dichotomous role of TGF- β in regulating iTreg cell fate through 2 distinct signal transduction pathways. One pathway was dependent on Smad3, leading to IL-10-producing suppressor iTreg cells, and a second was dependent on TGF- β -activated kinase 1 (TAK1) and IL-6, leading to IL-17-producing Teff cells and enhancement of lung allergic responses.

METHODS

Animals

Pathogen-free, 6- to 8-week-old female and male C57BL/6, CD90.1, and CD8^{-/-} mice (on a C57BL/6 background) were obtained from existing colonies at National Jewish Health, and additional C57BL/6 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 (Imject Alum; Pierce, Rockford, Ill) in a total volume of 100 μ L on days 1 and 14.⁵ Sensitized and challenged mice (OVA/OVA) and non-sensitized 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 using an ultrasonic nebulizer (Omron, Vernon Hills, Ill).

Measurement of airway responsiveness

Airway responsiveness was assessed as a change in airway function to aerosolized methacholine 48 hours after the last challenge.⁵ Methacholine was administered for 10 seconds (60 breaths/min, 500- μ L tidal volume) in increasing concentrations. Lung resistance was continuously computed (Labview; National Instruments, Austin, Tex) by fitting flow, volume, and pressure to an equation of motion. Maximum values of lung resistance were taken and expressed as a percentage change from baseline after PBS aerosol.

Bronchoalveolar lavage

Immediately after measurement of airway hyperresponsiveness (AHR), lungs underwent lavage.⁵ 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; Shandon, Runcorn, Cheshire, United Kingdom), stained with Leukostat (Fisher Diagnostics, Middletown, Va), and differentiated by using standard hematologic procedures. In some experiments cells from each animal group were pooled and investigated for staining of surface molecules by using FACS analysis.

Cell preparation and culture

CD4⁺CD25⁻ T cells were isolated from the spleens of naive C57BL/6 and CD90.1⁺ C57BL/6 mice. Lymphocytes were further magnetically sorted according to the manufacturer's instructions, resulting in a purity of 93% or greater CD4⁺CD25⁻ cells (CD4⁺CD25⁺ Regulatory T Cell Isolation Kit; all reagents were purchased from Miltenyi Biotec, Bergisch Gladbach, Germany). After depletion of CD4⁺CD25⁺ cells, CD4⁺CD25⁻ T cells in the negative fraction were reisolated by positive selection for CD4⁺ T cells, resulting in 98% or greater purity (CD4⁺ Microbeads). In some experiments transferred cells ($\geq 95\%$ of CD90.1⁺) were recovered from the lungs of CD90.2⁺ mice by means of positive selection after collagenase digestion.

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 FCS 10% (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; R&D Systems, Minneapolis, Minn) and stimulated with plate-bound anti-CD3 and soluble anti-CD28 (2 μ g/mL; eBioscience, San Diego, Calif) with or without TGF- β (2.5–5 ng/mL; R&D Systems) in combination with IL-6 (5 ng/mL; R&D Systems) or IL-6 alone for 7 days. Differentiated cells were washed, and 5×10^5 cells in 50 mL of PBS were adoptively transferred into sensitized recipients before allergen challenge. In some experiments TGF- β -cultured cells were treated during or after differentiation with a Smad3 (1 μ mol/L; SIS3) or TAK1 inhibitor (0.5 μ mol/L; 5Z-7-Oxozeaenol; Calbiochem, San Diego, Calif)²⁶ or mouse TAK1 or control short hairpin RNA (shRNA) lentivirus (Santa Cruz Biotechnology, Santa Cruz, Calif).

Measurement of cytokine levels

Cytokine levels in the bronchoalveolar lavage (BAL) fluid and supernatants of *in vitro*-cultured cells were measured by means of ELISA, including IL-5, IL-6, IL-10, IL-13, IL-17, IL-21, IL-22, IFN- γ , and TGF- β (eBioscience/Thermo Fisher Scientific, San Diego, Calif).⁵ ELISAs were performed according to the manufacturers' directions. The limits of detection were 4 pg/mL for IL-5, IL-6, and IL-17; 10 pg/mL for IL-10 and IFN- γ ; 12 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 eBioscience/Thermo Fisher Scientific: anti-CD3 fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridinin-chlorophyll-protein complex (PerCP), and allophycocyanin (APC; 17A2); CD4 FITC, PE, PerCP, and APC (L3T4); CD11b (M1/70) FITC, PE, and APC; CD25 FITC (7D4) and PE (PC61); anti-CD90.1 (HIS5), biotin; F4/80 (BM8) FITC, PE, and APC; and Ly-6G (Gr-1, RB6-8C5) FITC, PE, APC. For intracellular staining, cells were stimulated with phorbol 12-myristate 13-acetate (100 ng/mL; Sigma-Aldrich) and ionomycin (2 μ g/mL; Sigma-Aldrich) in complete medium overnight and for an additional 6 hours in the presence of brefeldin A (10 μ g/mL; Sigma-Aldrich). Cells were fixed with 4% formaldehyde in PBS, permeabilized in 0.5% saponin, and stained as follows: anti-Foxp3 PE and APC (FJK-16s); ROR γ t PE and APC (AFKJS-9) (eBioscience); phosphorylated Smad3 (C25A90; Cell Signaling Technology, Danvers, Mass); and TAK1 (H5; Santa Cruz Biotechnology). 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, San Jose, Calif) with CellQuest Pro software. Fluorescence intensity was compared with cells stained with corresponding labeled isotype-matched control antibodies.

Immunofluorescence staining

Intracellular expression of Smad3 and TAK1 in the same cell after culture in different conditions, as described in the section "*In vitro* differentiation and adoptive transfer" (TCR activation alone, with IL-6 or TGF- β or IL-6 and TGF- β), was analyzed by means of immunofluorescence staining. Cultured cells were stimulated with phorbol 12-myristate 13-acetate (100 ng/mL) and ionomycin (2 μ g/mL) in complete medium for 6 hours in the presence of brefeldin A (10 μ g/mL) before seeding on poly-D-lysine-coated coverslips. Cells were fixed and permeabilized for 1 hour, as described previously¹⁰; washed several times; and blocked with Superblock solution supplemented with 2% naive rat serum for 1 hour. Cells were then incubated with either anti-phosphorylated Smad3 (C25A90; Cell Signaling Technology) or anti-TAK1 (H5; Santa Cruz Biotechnology) overnight on a shaker at 4°C and then washed extensively for 2 hours. Coverslips were affixed with mount medium containing 4'-6-diamidino-2-phenylindole dihydrochloride (Vector Laboratories, Burlingame, Calif). Slides were analyzed with a Zeiss LSM 700 microscope and Zen Black Software (Zeiss, Thornwood, NY).⁵

Quantitative PCR

Total RNA was extracted from cells with the NucleoSpin RNA II Isolation Kit (Macherey-Nagel, Duren, Germany), according to the manufacturer's instructions. cDNA was synthesized with an equal amount of RNA (0.25 μ g from recovered cells and 1.0 μ g from *in vitro*-differentiated cells) by using the QuantiTect Reverse Transcription Kit (Qiagen, Valencia, Calif). Predesigned primers and probes for mouse *Il10* (Mm00439614_m1), *Il17* (Mm00439618_m1), *Foxp3* (Mm00475162_m1), *Roryt* (Mm001261022_m1), *Tak1* (Mm00554514_m1), and *Gapdh* (Mm99999915_g1) were obtained from Applied Biosystems (Grand Island, NY). PCR was performed with the ABI Prism 7500 Sequence Detection System. Analyses of relative gene expression were carried out with the mathematic calculation model based on $\Delta\Delta$ cycle threshold values.⁵

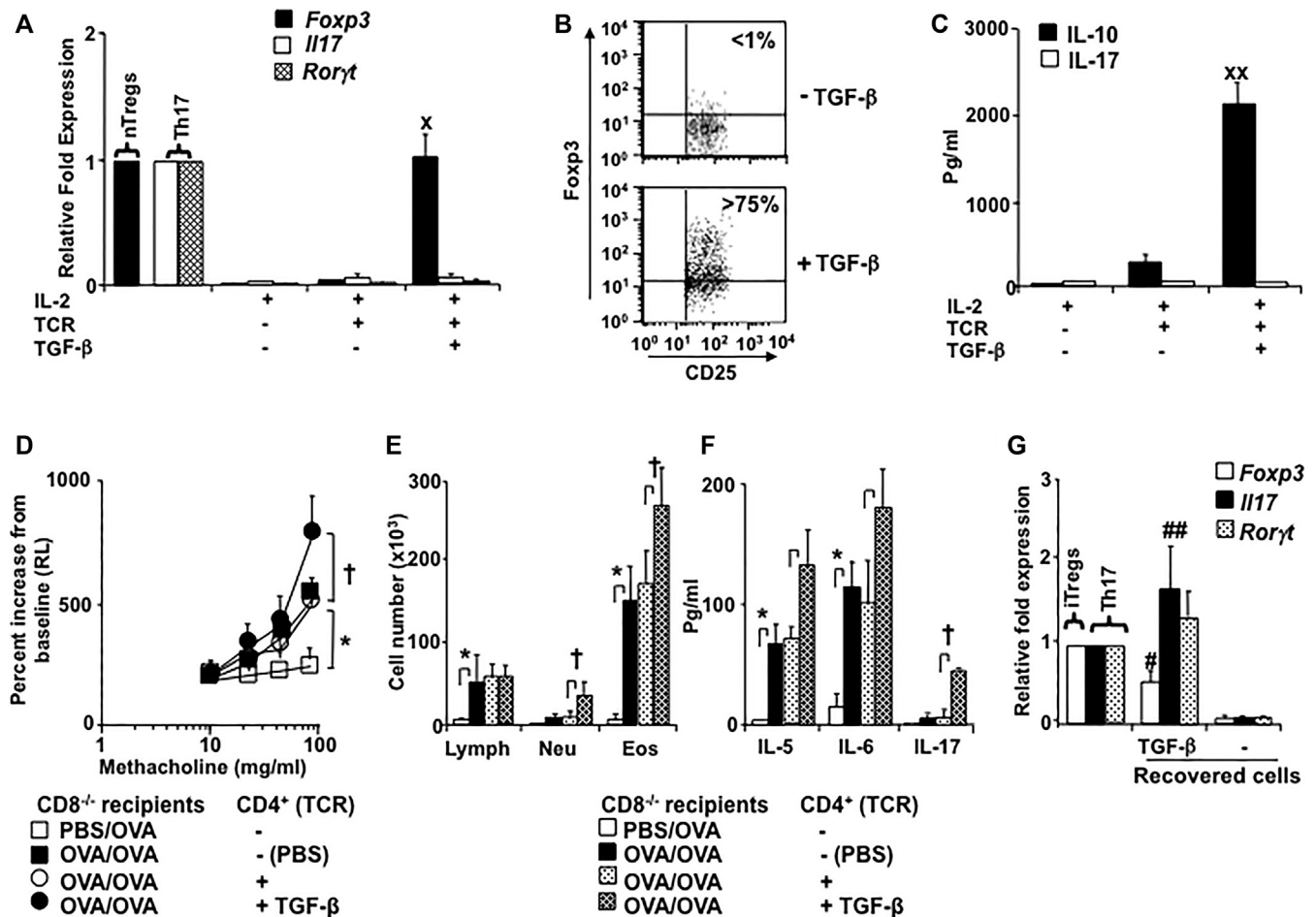


FIG 1. Transfer of iTreg cells enhances lung allergic responses in sensitized and challenged CD8^{-/-} recipients. **A**, Quantitative gene expression (quantitative PCR). **B**, FACS analysis after TCR activation with or without TGF- β . **C**, Cytokine production of naive CD4⁺CD25⁺ T cells after culture with IL-2 alone or with anti-CD3/CD28 or TGF- β for 5 days. **D**, AHR. **E**, BAL fluid inflammatory cell composition. *Eos*, Eosinophils; *Lymph*, lymphocytes; *Neu*, neutrophils. **F**, BAL fluid cytokine levels. **G**, Quantitative gene expression levels of transferred cells recovered from lungs of recipients. Shown are means $\pm$ SEMs from 3 independent experiments (4 mice per group, n = 12). X Gene expression levels or XX Cytokine levels: P < .01 comparing naive CD4⁺ T cells and TCR-activated cells with iTreg cells (taking expression levels of *Foxp3*, *Il17*, and *Roryt* in cells before transfer as 1). *P < .05 comparing sensitized and challenged mice with mice challenged only. †P < .05 comparing sensitized and challenged recipients of iTreg cells with recipients of TCR-activated CD4⁺ T cells. #P < .05 comparing expression levels of *Foxp3* in recovered and transferred cells to Treg cells before transfer. ##P < .05 comparing expression levels of *Roryt* and *Il17* in recovered transferred cells with T_H17 cells before transfer (taking expression levels of iTreg or T_H17 cells before transfer as 1).

Chromatin immunoprecipitation

Chromatin immunoprecipitation assays of TCR-activated cells (5×10^6) in the presence of TGF- β alone (iTregs) or with IL-6 (T_H17) were performed on day 7 according to the manufacturer's protocol by using the ChIP IT Express Kit (Active Motif, Carlsbad, Calif) and as previously published.²⁷ Briefly, chromatin was cross-linked for 7 minutes at room temperature by adding 1% methanol-free formaldehyde (Thermo Fisher, Waltham, Mass). Cross-linking was stopped by the addition of 1 $\times$ glycine-stop fix solution at room temperature. After incubation of cells for 30 minutes at 4°C, cell nuclei were pelleted and resuspended in 130 μ L of shearing buffer containing protease inhibitors. Shearing was done with an optimized 23-cycle treatment with a focused energy isothermal sonicator (Covaris S2 sonicator; Covaris, Woburn, Mass), providing DNA fragments with an average size of 300 to 500 bp, with the highest density at 500 bp. Supernatant (5 μ L) was saved for use as total input DNA.

Immunoprecipitations were performed according to manufacturers' protocols with protein G magnetic beads overnight at 4°C, with an estimated 2 μ g of sheared chromatin. Equal amounts of chromatin were used for the immunoprecipitations with 6 μ g of anti-H3K4me3-specific (07-473; Millipore, Burlington, Mass) and H3K27me3-specific antibodies (ABE44; Millipore). After washing and eluting the beads with respective kit buffers, reverse cross-linking was performed for 2 hours at 95°C, followed by a proteinase K treatment (10 μ g/mL) for 1 hour at 37°C. The immunoprecipitated genomic DNA was purified with the Qiagen MinElute Reaction Cleanup Kit (Qiagen) and analyzed through SYBR green (SYBR Green Select Mastermix; Life Technologies, Carlsbad, Calif) quantitative PCR with primers for *Roryt* (see Table E1 in this article's Online Repository at www.jacionline.org). Changes in enrichment of histone marks were determined by targeting approximately 2000 bp upstream of the transcription start site of *Roryt* (Ensemble Genome browser, ENSMUST00000197040,

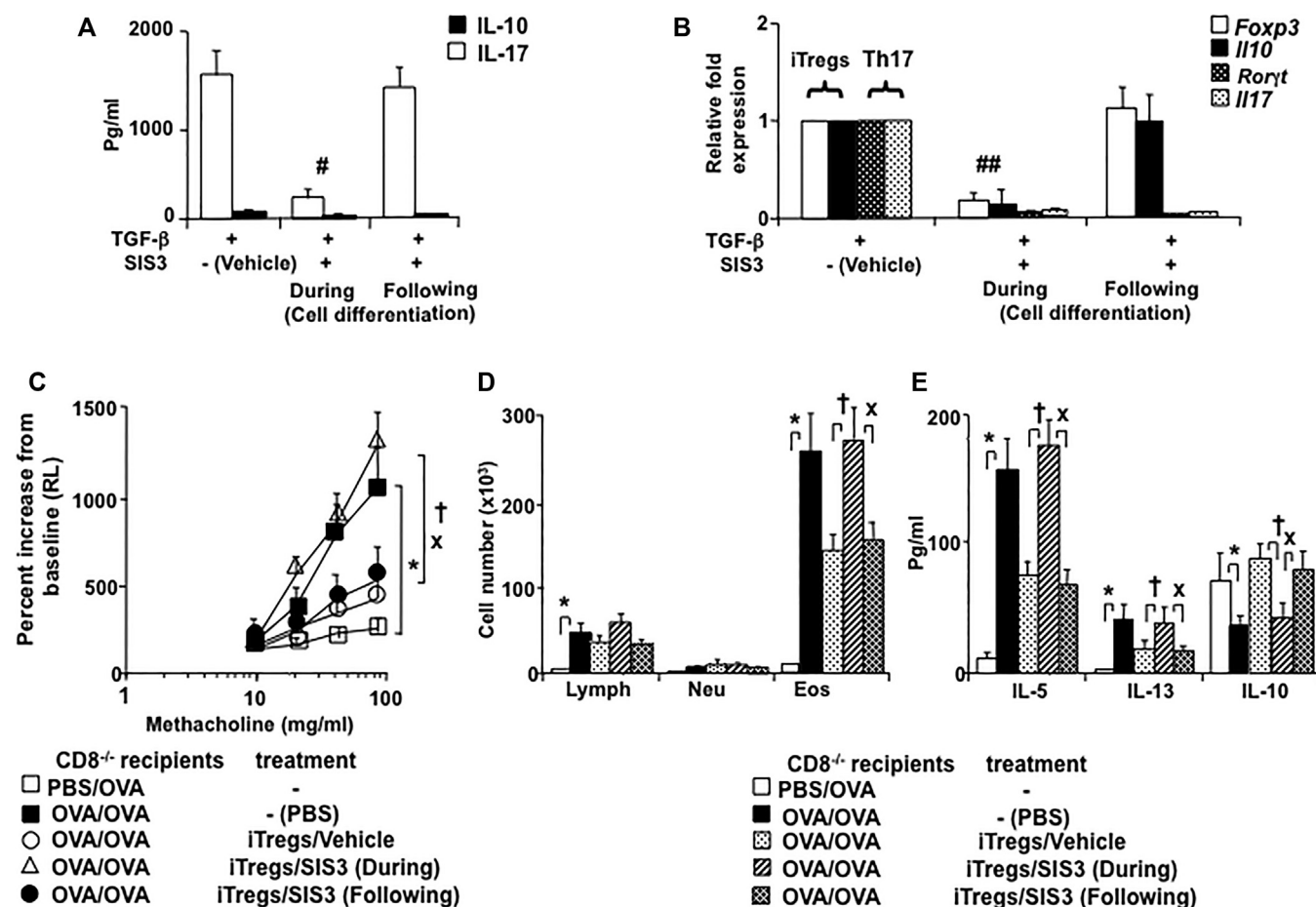


FIG 2. Inhibition of Smad3 activation during cell differentiation attenuates iTreg cell-mediated suppression of lung allergic responses in sensitized and challenged WT (CD8^{+/+}) mice. **A**, Effect of SIS3 on *in vitro* production of cytokines. **B**, Quantitative gene expression levels of cultured cells in the presence of SIS3 during or after cell differentiation. **C**, AHR. **D**, BAL fluid inflammatory cell composition. *Eos*, Eosinophils; *Lymph*, lymphocytes; *Neu*, neutrophils. **E**, BAL fluid cytokine levels. Shown are means $\pm$ SEMs from 3 independent experiments (4 mice per group, $n = 12$). #Cytokine and ##gene expression levels (taking expression levels of iTreg or Th17 cells as 1): $P < .05$ comparing TGF- β -exposed cells treated with vehicle or SIS3 during or following 7-day differentiation; cell differentiation (taking the expression levels of iTreg or Th17 cells as 1). * $P < .01$ (AHR, eosinophils, and IL-5) and * $P < .05$ (lymphocytes, IL-13, and IL-10) comparing sensitized and challenged mice with mice challenged only. † $P < .05$ comparing recipients of cells exposed to TGF- β treated with vehicle to SIS3 during or after cell differentiation. XP $P < .05$ comparing recipients of cells exposed to TGF- β treated with SIS3 during differentiation with cells treated after cell differentiation.

Rorγt_1, *Rorγt_2*, *Rorγt_3*, and *Rorγt_4*). The location of the 4 overlapping primer pairs spanned approximately 500 bp each (Fig 8, A, and see Table E1).

Statistical analysis

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

RESULTS

TGF- β -polarized CD4⁺ T cells enhance lung allergic responses in sensitized and challenged CD8^{-/-} mice

Initially, the requirement for TGF- β was established in the generation of iTreg cells by determining *in vitro* expression levels

of the lineage-specific transcription factor Foxp3 and production of IL-10 and demonstrating the unique role of iTreg cells in the enhancement of lung allergic responses. Although TCR activation (anti-CD3/CD28) of CD4⁺CD25⁻ cells alone increased expression of the low-affinity IL-2 receptor α -chain (CD25), it was insufficient to induce expression of Foxp3 (Fig 1, A and B) or significant levels of IL-10 (Fig 1, C). In contrast, cells activated through the TCR together with TGF- β (iTreg cells) expressed Foxp3 mRNA and protein, as determined by using quantitative PCR and flow cytometry (FACS), as well as production of IL-10 (Fig 1, A-C). These cells were potent suppressors of the development of lung allergic responses in WT recipients.⁵ Cells cultured under these conditions did not express *Rorγt* or produce IL-17 (Fig 1, A and C).

Unlike WT mice,^{5,10} CD8^{-/-} mice had limited lung allergic responses after allergen sensitization and challenge (Fig 1, D-F). Increases in numbers of lymphocytes and eosinophils in

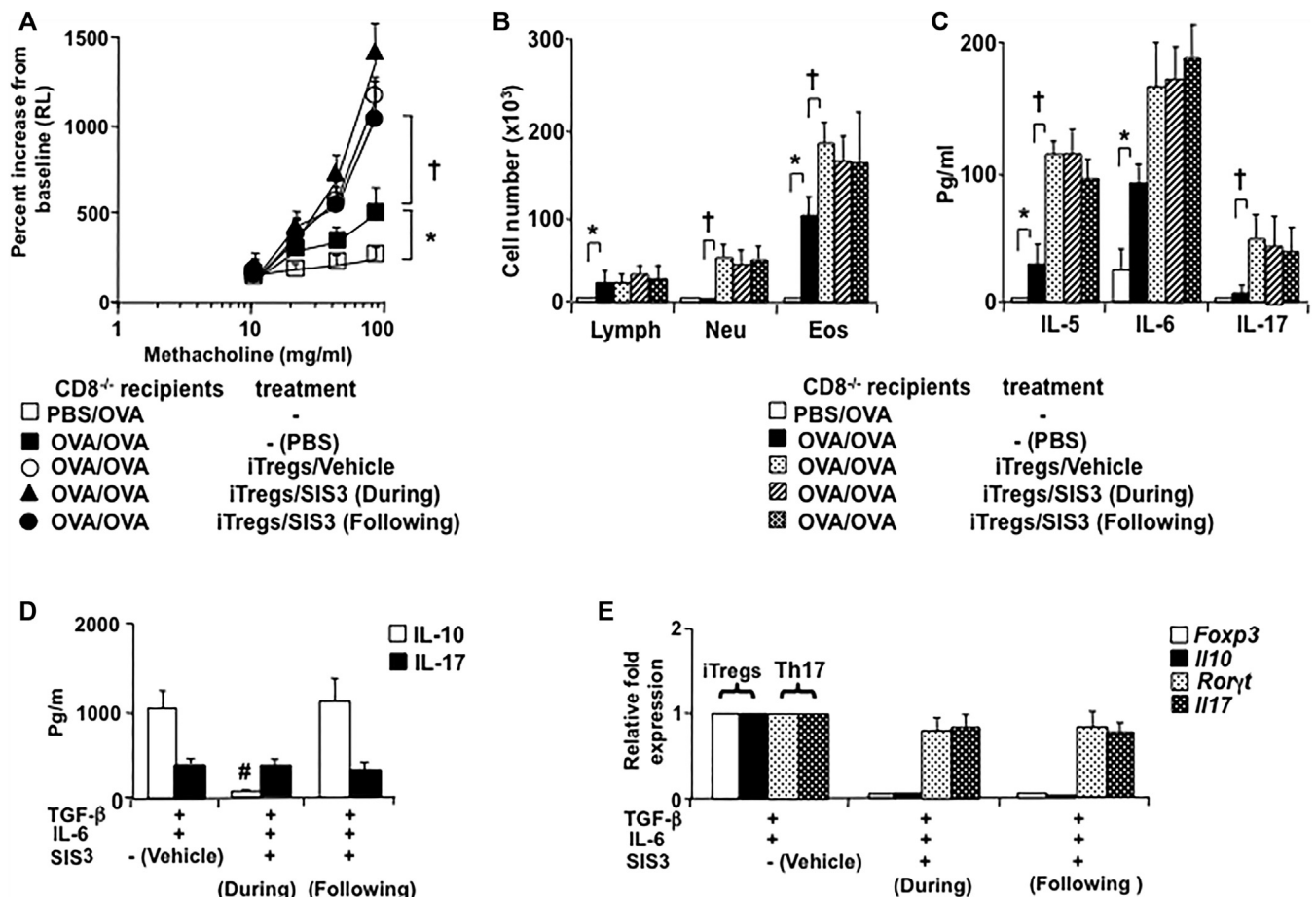


FIG 3. Inhibition of Smad3 activation during or after cell differentiation does not alter iTreg cell effector function in sensitized and challenged CD8^{-/-} recipients. **A**, AHR. **B**, BAL fluid inflammatory cell composition. **C**, BAL fluid cytokine levels. **D**, Effect of SIS3 on *in vitro* production of cytokines. **E**, Effect of SIS3 on gene expression. Shown are means $\pm$ SEMs from 3 independent experiments (4 mice per group, $n = 12$). * $P < .05$ comparing sensitized and challenged mice with mice challenged only. † $P < .05$ comparing sensitized and challenged CD8^{-/-} mice with recipients of cells exposed to TGF- β treated with vehicle or SIS3 during or after cell differentiation. # $P < .05$ comparing TGF- β - and IL-6-exposed cells treated with vehicle with SIS3 during or after cell differentiation.

association with increased levels of IL-5 and IL-6 but little change in IL-17 levels in BAL fluid after sensitization and challenge were similar to levels seen in CD8^{-/-} recipients given cells that were TCR-activated alone (without TGF- β , Foxp3⁺CD4⁺ T cells). In contrast, transfer of TGF- β -induced iTreg cells (Foxp3⁺CD4⁺ T cells) into sensitized and challenged CD8^{-/-} recipients resulted in increased AHR and numbers of eosinophils and neutrophils and increased levels of IL-5, IL-6, and IL-17 in the BAL fluid (Fig 1, D-F).

In parallel to the changes in lung physiology and inflammation, significantly increased *Roryt* and *Il17* gene expression and decreased *Foxp3* mRNA levels were detected in recovered cells from the lung when compared with levels seen in cells before transfer (Fig 1, G). None of these findings were observed in recovered cells from the lungs of CD8^{-/-} recipients that received cells that were only TCR activated before transfer. Also, enhancement of lung allergic responses was demonstrated to be dependent on *in vitro* differentiation with TGF- β in a dose-dependent fashion (see Fig E1 in this article's Online Repository at www.jacionline.org). Thus TGF- β endowed iTreg cells with the capacity to undergo transcriptional reprogramming in sensitized and

challenged CD8^{-/-} recipients, resulting in enhancement of lung allergic responses.

Smad3 regulates the suppressive activities of iTreg cells but does not influence iTreg cell-mediated enhancement

TGF- β regulates Foxp3 expression through Smad3.^{20,24,25} The role of Smad3 on suppressor and effector function of iTreg cells during and after the 7-day cell differentiation period was investigated by using the specific Smad3 inhibitor SIS3.²⁸ Exposure to SIS3 during the entire 7-day duration of cell differentiation significantly reduced IL-10 mRNA and protein expression in association with decreased *Foxp3* expression (Fig 2, A and B). After adoptive transfer into sensitized and challenged WT recipients, these cells did not suppress the development of lung allergic responses (Fig 2, C-E). Treatment with SIS3 for 6 hours after the 7-day differentiation period, unlike inclusion of the inhibitor during differentiation, had limited effect on the capacity of iTreg cells to suppress lung allergic responses in the sensitized and challenged WT recipients. These results suggest that development of

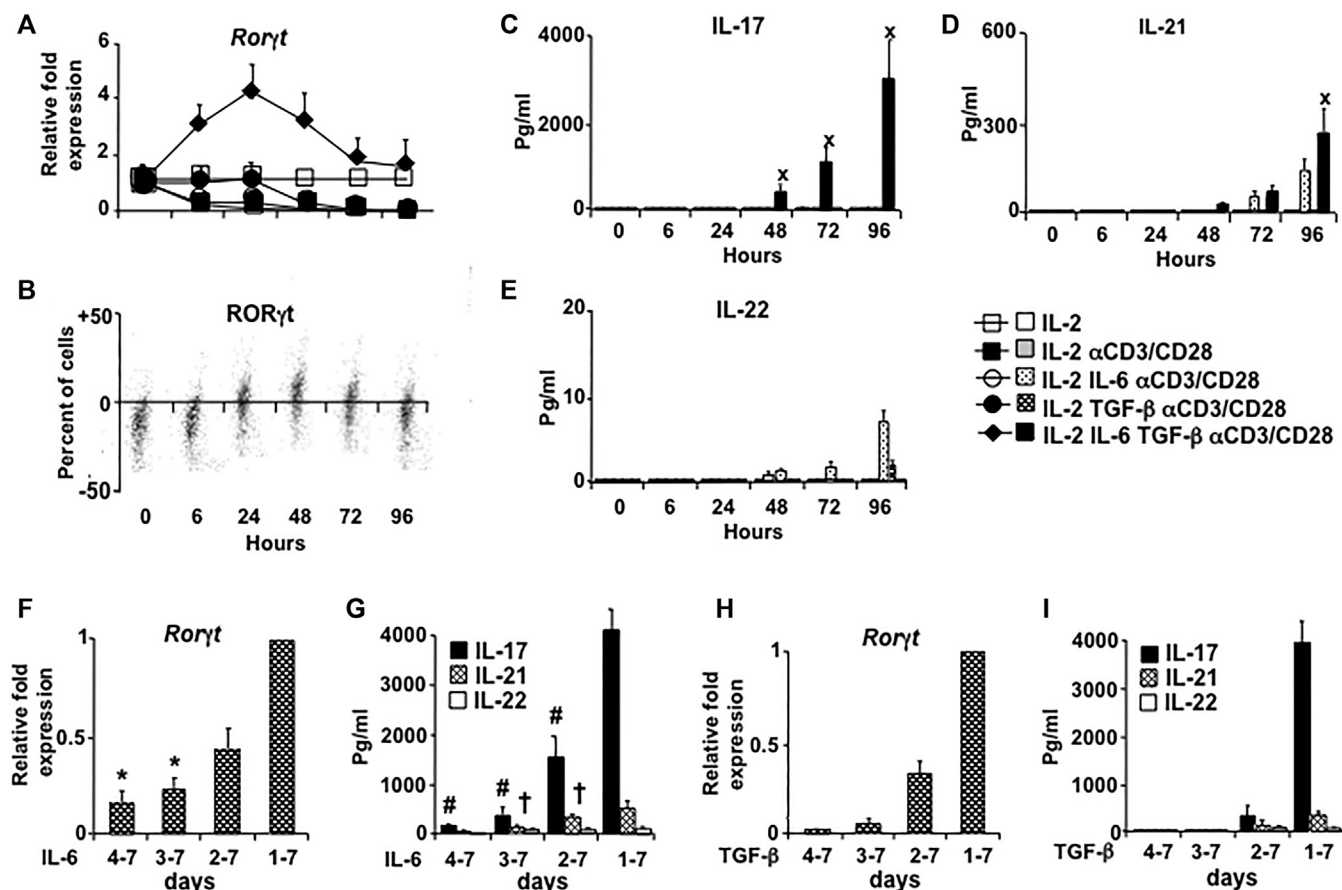


FIG 4. Order of exposure to TGF- β and IL-6 influences development of T_H17 cells and kinetics of gene and protein expression and cytokine production. **A**, *Roryt* gene expression levels. **B**, Percentage of $ROR\gamma t^+$ cells. **C-E**, Production levels of IL-17 (Fig 4, C), IL-21 (Fig 4, D), and IL-22 (Fig 4, E). Kinetics of expression of *Roryt* (Fig 4, F) and IL-17, IL-21, and IL-22 (Fig 4, G) and *Roryt* (Fig 4, H) and IL-17, IL-21, and IL-22 (Fig 4, I) depending on the order and timing of TGF- β or IL-6 addition, respectively. Results shown are means $\pm$ SEMs from 3 independent experiments. $XP < .05$ (IL-17, IL-21, and IL-22) comparing TCR-activated cells cultured with TGF- β (5 ng/mL) and IL-6 (5 ng/mL) with naive $CD4^+$ T cells, TCR activation alone or with IL-6 or TGF- β at each time point. $*P < .05$ (*Roryt*), $\#P < .05$ (IL-17), and $\dagger P < .05$ (IL-21) comparing TCR-activated cells exposed initially to TGF- β and then IL-6 (Fig 4, F and G) with cells exposed initially to IL-6 and then TGF- β (Fig 4, H and I) at the same time point.

the regulatory phenotype and suppressor function of iTreg cells was critically dependent on initial signaling by TGF- β through Smad3.

In contrast to the role of Smad3 on iTreg cell suppressor function in WT recipients, treatment with SIS3 neither during nor after cell differentiation had any effect on the enhancement of AHR, airway neutrophilia, or BAL fluid IL-17 in sensitized and challenged $CD8^{-/-}$ recipients (Fig 3, A-C). SIS3-treated cells were fully capable of producing IL-17 accompanied by significant increases in *Roryt* and *Il17* mRNA expression after exposure to IL-6 *in vitro* (Fig 3, D and E). Taken together, the data demonstrated that although required for induction of both iTreg cell-mediated suppression and iTreg cell-mediated T_H17 enhancement, TGF- β triggered these activities through distinct signaling pathways, one Smad3 dependent and the other Smad3 independent.

TGF- β is the initiating signal in induction of T_H17 differentiation

As previously shown, host-derived IL-6 in the lungs of sensitized and challenged $CD8^{-/-}$ recipients was essential to the *in vivo* reprogramming of iTreg cells to IL-17-producing $CD4^+$ Teff cells, resulting in enhancement of lung allergic responses.⁵ Here the role of IL-6 alone on differentiation, expression of the lineage-specific transcription factor *ROR γt* , *in vitro* production of T_H17 -related cytokines (IL-17, IL-21, and IL-22), and effector function was further characterized. IL-6 alone was not sufficient to induce expression of *ROR γt* mRNA or protein (Fig 4, A, and see Figs E2, C, and E3, B, in this article's Online Repository at www.jacionline.org) or able to generate T_H17 cells producing IL-17, IL-21, or IL-22 in TCR-activated cells both *in vitro* and *in vivo* (Fig 4, C-E, and see Figs E2, F, and E3, A). Transfer of TCR-activated $CD4^+$ T cells cultured with IL-6 alone

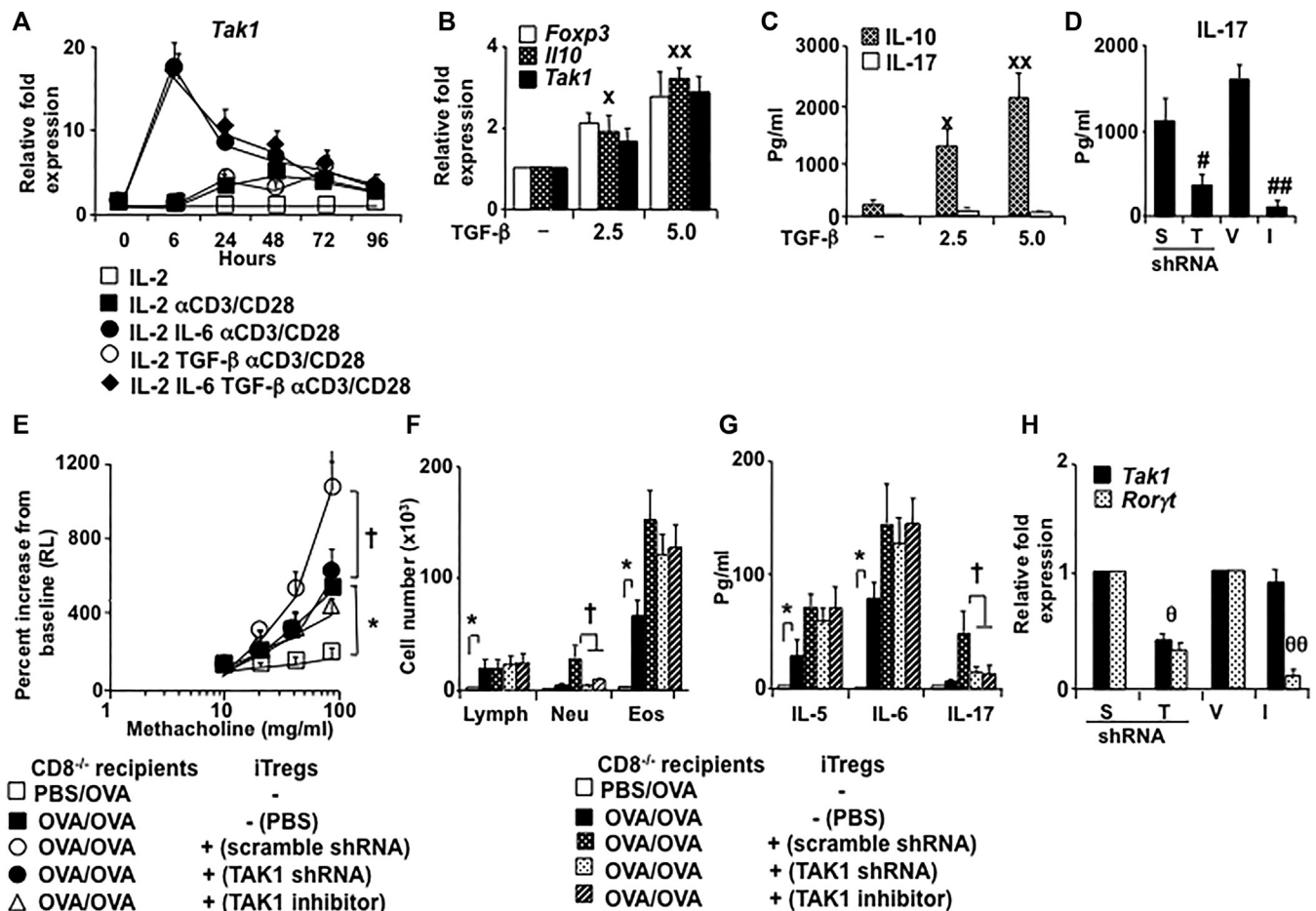


FIG 5. TGF- β regulates kinetics and dose dependence of *Tak1* expression and cytokine production; TAK1 inhibition decreases IL-17 and prevents iTreg cell-mediated enhancement of lung allergic responses in sensitized and challenged CD8^{-/-} recipients. **A**, Time-dependent gene expression. **B**, TGF- β dose-dependent gene expression. **C**, TGF- β dose-dependent cytokine production. **D**, Effect of TAK1 inhibition on IL-17 production. **E**, AHR. **F**, BAL fluid cell composition. *Eos*, Eosinophils; *Lymph*, lymphocytes; *Neu*, neutrophils. **G**, BAL fluid cytokine levels. **H**, Gene expression of transferred cells recovered from lungs of CD8^{-/-} recipients. Shown are means $\pm$ SEMs from 3 independent experiments (4 mice per group, $n = 12$). $P < .05$ comparing gene and cytokine levels of 2.5 ng/mL TGF- β to TCR activation alone. $XXP < .05$ comparing gene expression and cytokine production in TCR-activated cells exposed to 2.5 or 5 ng/mL TGF- β . $\#P < .05$ comparing cells transfected with *Tak1* (T) to scrambled shRNA (S). $\#\#P < .05$ comparing cells treated with TAK1 inhibitor (I) to vehicle (V). $*P < .05$ comparing sensitized and challenged CD8^{-/-} mice with mice challenged only. $\dagger P < .05$ comparing recipients of cells treated with scrambled shRNA to cells treated with *Tak1* shRNA or inhibitor. $\theta P < .05$ comparing recovered cells from CD8^{-/-} mice that received cells transfected with *Tak1* (T) to scrambled shRNA (S) before transfer. $\theta\theta P < .05$ comparing recovered and transferred cells treated with TAK1 inhibitor (I) or vehicle (V) before transfer (taking expression of cells treated with scrambled shRNA or vehicle as 1).

into CD8^{-/-} recipients did not enhance lung allergic responses, identifying the critical initiating role of TGF- β in T_H17 development and function (see Fig E3, C-E).

Because both TGF- β and IL-6 were required for generation of T_H17 cells both *in vitro* and *in vivo*, the sequential importance of TGF- β and IL-6 signaling in iTreg cell reprogramming leading to IL-17 production was further investigated in cultured cells. TCR-activated cells were continuously exposed (from day 1) to either IL-6 or TGF- β over the 7-day differentiation period. After initiation of the cultures, on days 2, 3, or 4, TGF- β (5 ng/mL) was added to cultures that contained IL-6 or IL-6 (5 ng/mL) was added to cultures that contained TGF- β for the remainder of the 7-day culture period. Expression levels of the transcriptional

regulators *Foxp3* and *Roryt* and the associated cytokines IL-10, IL-17, IL-21, and IL-22 were compared under each condition. Early induction of *Foxp3* (6-24 hours) and gradual increases in IL-10 gene and protein expression (48-96 hours) were detected in TCR-activated cells exposed to TGF- β (see Fig E2, A-C). Neither TGF- β alone nor IL-6 alone induced significant increases in gene expression levels of *ROR γ t* or IL-17 gene and protein expression over the 7-day time course (see Fig E2, D-F). In contrast, cells differentiated *in vitro* in the presence of both TGF- β and IL-6 expressed *ROR γ t* mRNA and protein, with the highest detectable levels at the midpoint of the time course (24-48 hours), and produced IL-17 and IL-21 (but little IL-22) in increasing amounts over time (Fig 4, A-E).

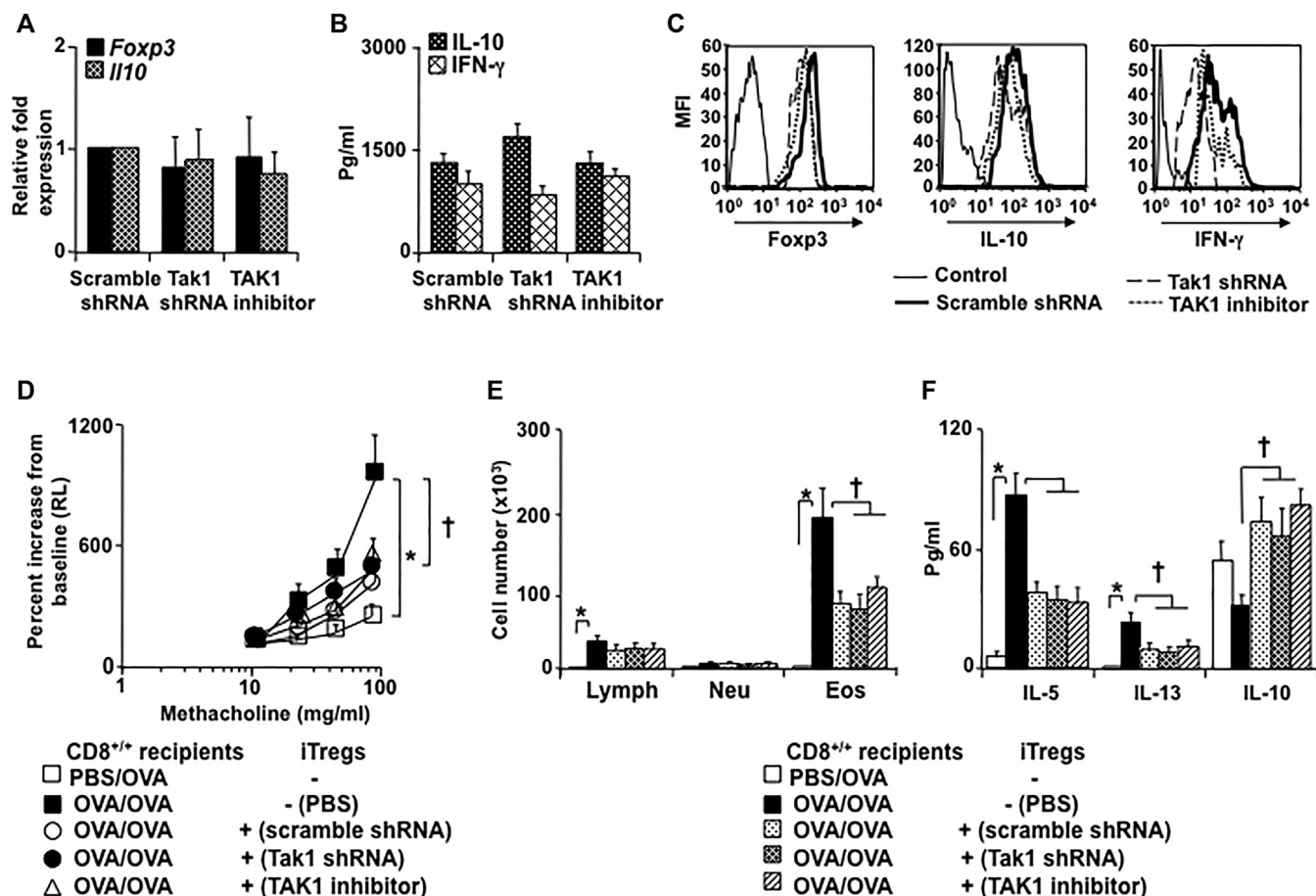


FIG 6. TAK1 attenuation using shRNA or an inhibitor does not alter *in vitro* development of iTreg cells or iTreg cell-mediated suppression of lung allergic responses in sensitized and challenged WT (CD8^{+/+}) recipients. **A**, Quantitative gene expression levels of cells after gene silencing or inhibition of enzymatic activity (taking expression of cells treated with scramble shRNA as 1). **B**, *In vitro* production of cytokines in cells treated with scrambled or Tak1 shRNA or inhibitor. **C**, Intracellular staining of proteins by using flow cytometry (FACS). **D**, AHR. **E**, BAL fluid inflammatory cell composition. *Eos*, Eosinophils; *Lymph*, lymphocytes; *Neu*, neutrophils. **F**, BAL fluid cytokine levels. Shown are means $\pm$ SEMs from 3 independent experiments (4 mice per group, $n = 12$). * $P < .01$ (AHR, eosinophils, and IL-5) and * $P < .05$ (lymphocytes, IL-13, and IL-10) comparing sensitized and challenged mice with mice challenged only. † $P < .05$ comparing sensitized and challenged mice with recipients of cells treated with scrambled or Tak1 shRNA or inhibitor.

Cells initially exposed to TGF- β with IL-6 added to the cultures at later time points (days 2, 3, or 4) produced significant amounts of IL-17 and IL-21 together with early expression of *Roryt*, which increases over time (Fig 4, F and G). In contrast, in cells initially exposed to IL-6 but with TGF- β added to the cultures at later time points (days 2, 3, or 4), comparatively little IL-17 and IL-21 were detected, and limited induction of *Roryt* mRNA was demonstrated (Fig 4, H and I). These data identified a hierarchy of signaling events for effective differentiation of T_H17 cells and the initial importance of TGF- β signaling.

Signal transduction by TGF- β through TAK1 regulates T_H17 differentiation and transcriptional reprogramming of iTreg cells into IL-17-producing Teff cells

Because inhibition of Smad3 only affected suppressor function of iTreg cells whereas effector function was unaffected, this suggested divergent signaling pathways downstream of the

common requirement for TGF- β . Activation of TAK1 (MAP3K7) in response to various factors, including TGF- β , has been shown to play important roles in innate immunity and in proinflammatory signaling pathways.^{29,30} We determined whether TAK1 activation also played a role in functional reprogramming of iTreg cells from suppressor to IL-17⁺ Teff cells. Induction of *Tak1* gene expression at an early time point (6 hours) was detected in activated CD4⁺ T cells cultured with TGF- β alone and was TGF- β dose dependent in a similar fashion to induction of *Foxp3* and *Il10* (Fig 5, A and B). Induction of *Tak1* gene expression was independent of IL-6 (Fig 5, A). Corresponding increases in IL-10, but not IL-17, protein levels in the absence of IL-6 were detected in cells exposed to increasing amounts of TGF- β (Fig 5, C). Inhibition of TAK1 either by blocking enzymatic activation with (5Z)-7-oxozeaenol, a selective TAK1 inhibitor,²⁶ or gene silencing with *Tak1*-specific shRNA was effective in significantly reducing *in vitro* production of IL-17 in cells differentiated under T_H17 conditions (TGF- β /IL-6; Fig 5, D). In parallel, both treatments (inhibitor or gene silencing) of TGF-

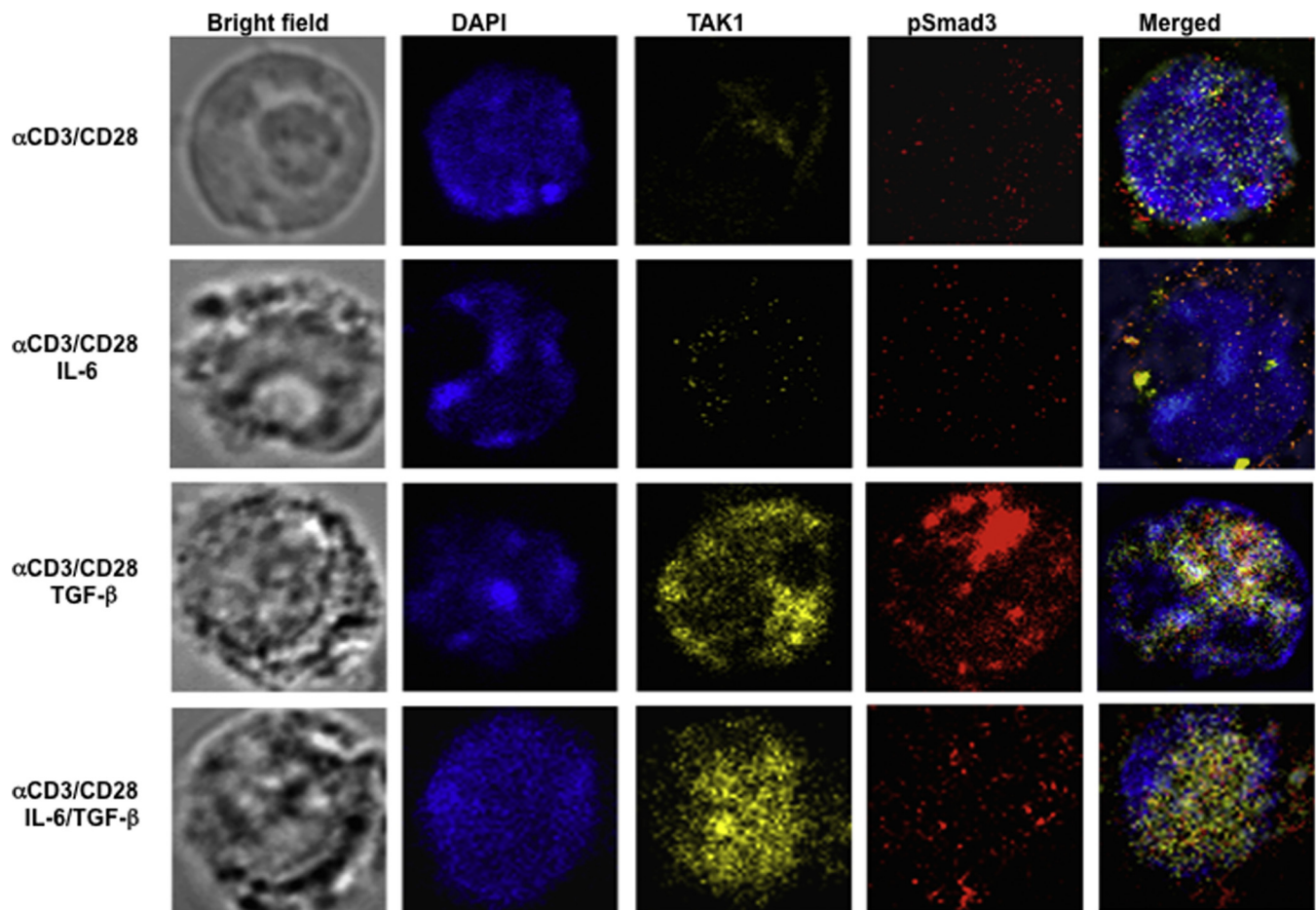


FIG 7. TGF- β induces TAK1 and Smad3 protein expression in the same cell, whereas IL-6 decreases Smad3 but not TAK1 expression. Representatives of intracellular immunofluorescence staining of cells cultured under different conditions. *Bright field*, Cell morphology and integrity; 4'-6-diamidino-2-phenylindole dihydrochloride (*DAPI*), nuclear staining; *yellow*, TAK1 proteins detected by a specific antibody; *red*, activated Smad3 protein detected by a specific antibody; *Merged*, Qualitative distribution and colocalization of TAK1 and pSmad3 proteins.

β -treated cells were equally effective in attenuating iTreg cell-mediated enhancement of lung allergic responses after transfer of treated cells into sensitized and challenged CD8^{-/-} recipients (Fig 5, E-G).

Corresponding to these functional outcomes in recovered cells from the lungs of recipients of TAK1-inhibited (through shRNA or inhibitor) cells, *Roryt* mRNA expression was significantly lower than in cells recovered from recipients of vehicle-treated or scrambled shRNA-manipulated cells (Fig 5, H). Expression of *Tak1* mRNA was unaffected in cells treated with the inhibitor because only enzymatic activation of TAK1 was blocked. Hence attenuation of TAK1 with inhibitor or shRNA prevented development of IL-17-producing cells and enhancement of lung allergic responses.

TAK1 is not essential for programming of iTreg cells and suppression of lung allergic responses

In contrast to interference with Smad3 activation in iTreg cells, inhibition of TAK1 with the inhibitor or shRNA had little effect

on iTreg cell expression of mRNA of *Foxp3* and *Il10* mRNA, *in vitro* production of IL-10 and IFN- γ , or intracellular protein staining by using FACS (Fig 6, A-C). Correspondingly, these treated cells retained suppressor activity to prevent development of lung allergic responses, with reduction of AHR, inflammation, and T_H2 cytokine skewing in sensitized and challenged WT recipients (Fig 6, D-F). These data further confirmed the independence of the 2 TGF- β -dependent signaling pathways.

Given the pivotal role of TGF- β in inducing 2 distinct pathways, one Smad3 dependent and the other TAK1 dependent, using immunofluorescence staining, we determined whether the same cell coexpressed both Smad3 and TAK1 proteins after TGF- β activation. In activated cells cultured with TGF- β but not cells cultured with IL-6 or TCR activation alone, both TAK1 and Smad3 proteins were detected in the same cell (Fig 7). When IL-6 was added to these cells, less robust staining for Smad3 was detected, whereas TAK1 expression was maintained. Thus, coexpression of both proteins in the same cell confirmed the dichotomous role of TGF- β in development of either iTreg or T_H17 cells.

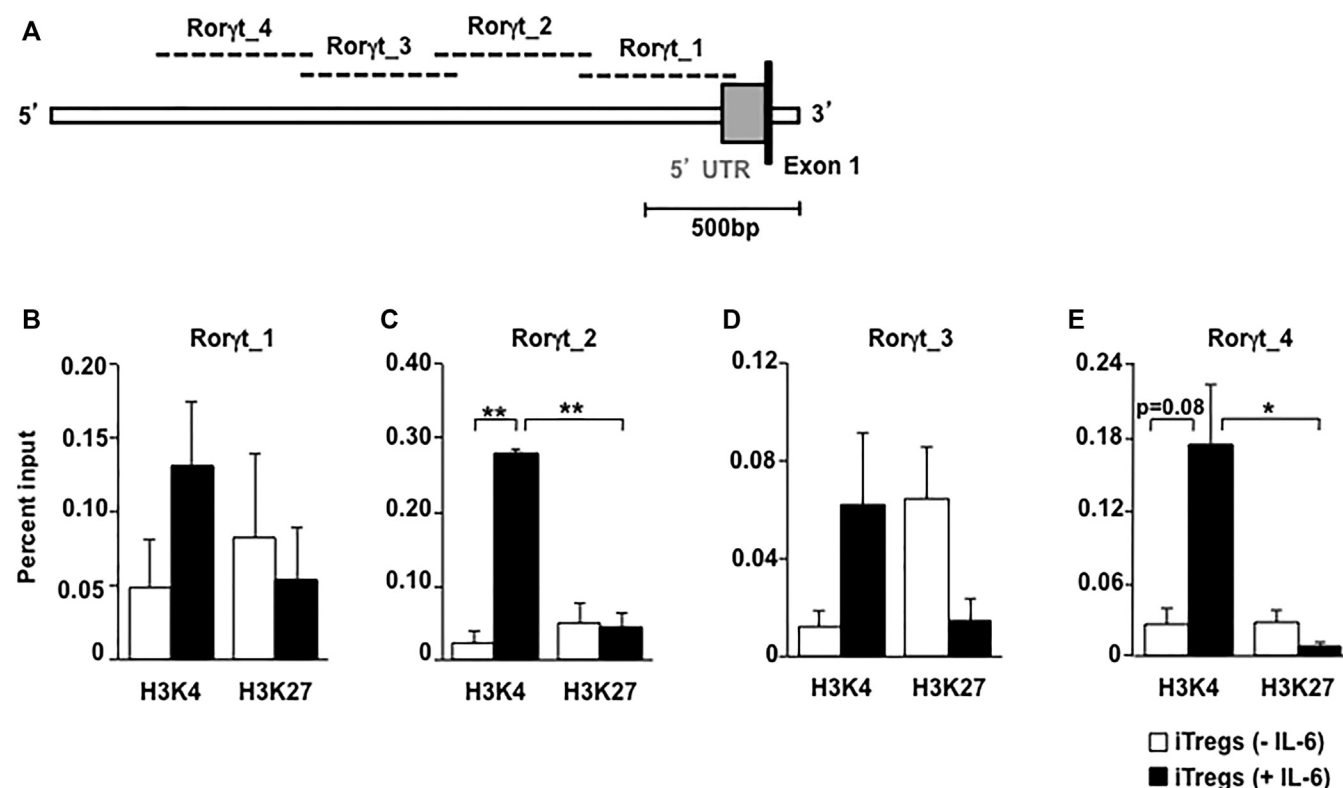


FIG 8. Exposure to TGF- β alone or together with IL-6 results in enrichment of H3K4me3 and reduction of H3K27me3 within the *Roryt* promoter. **A**, Schematic of the mouse *Roryt* promoter region based on Ensemble Genome browser data (ENSMUST00000197040) highlighting the region (2000 bp upstream of the transcription start site) investigated for histone modifications using 4 overlapping primer pairs (see Table E1), each spanning approximately 500 bp. Chromatin immunoprecipitation assays were performed with cells exposed to TGF- β (5 ng/mL) alone for 7 days or with IL-6 (5 ng/mL) from days 5 to 7 after differentiation with TGF- β . DNA was immunoprecipitated by using H3K4me3 or H3K27me3 antibodies. **B-E**, Precipitated DNA was quantified by using quantitative PCR. Data were analyzed by using percentage input methodology as follows: $2^{-(\text{Cycle threshold of total input} - \text{Cycle threshold of specific IP})}$. Data (relative fold change + SEM) are from 3 independent experiments. Pairwise comparisons were performed by using *t* tests: * $P < .05$ and ** $P < .01$.

Histone modifications at the *Roryt* locus determine the plasticity and cell fate of iTreg cells

Given the initial signaling requirements through TGF- β for susceptibility to IL-6-mediated T_H17 differentiation and increased expression of ROR γ t, changes in trimethylation (me3) of the histone proteins H3K4 (H3K4me3, gene activation) and H3K27 (H3K27me3, gene repression) in the regulatory regions of *Roryt* (Fig 8, A) were investigated. Compared with TGF- β alone, cells exposed to TGF- β and IL-6 had significant increases in H3K4me3 modifications in *Roryt* promoter regions 2 and 4 (Fig 8, C and E). In addition, enrichment of H3K4me3 in the same regions was inversely correlated with levels of H3K27me3 in iTreg cells differentiated in TGF- β and IL-6 (Fig 8, C and E). A similar trend was observed for regions 1 and 3 of the *Roryt* promoter, although this did not reach statistical significance (Fig 8, B and D). Unlike the results for the *Roryt* promoter in cells exposed to TGF- β and IL-6, histone methylation status of H3K4me3 and H3K27me3 in iTreg cells (TGF- β alone) did not show marked differences in the promoter region or 2 conserved noncoding sequences (CNSs; CNS1 and CNS2)^{31,32} of the *Foxp3* locus (data not shown). Taken together, the data identify that histone modifications of specific regions in the *Roryt*

promoter in cells differentiated with TGF- β represent an important mechanism regulating the conversion of iTreg cells into IL-17-producing Teff cells in response to IL-6.

DISCUSSION

In the present study we investigated the pivotal role of TGF- β and the hierarchy of signaling events resulting in 2 separate functional signal transduction pathways determining the functional outcomes of iTreg cells on lung allergic responses (see the Graphical Abstract). Early signaling events triggered by TGF- β through a Smad3-dependent cascade were critical to the development of suppressor iTreg cells through regulation of Foxp3 expression. This pathway was dispensable for T_H17 cell programming. A second pathway dependent on TAK1 signaling was essential for the development of T_H17 cells and the transcriptional reprogramming of iTreg cells into IL-17⁺ Teff cells. Identification of these 2 independent pathways, the dichotomous role of TGF- β acting in the same cell, and the novel and essential role of TAK1 in T_H17 generation was facilitated by using CD8^{-/-} mice as recipients. TGF- β /TAK1-dependent signaling was not required for generation of suppressor iTreg cells, whereas

Smad3 was not involved in TGF- β /TAK1-dependent T_H17 development. Conversion of iTreg cells into IL-17⁺ Teff cells was associated with histone modifications at the promoter of the lineage-specific transcription factor *Ror γ t*.

As previously shown,^{5,11,14,15} sensitized and challenged CD8^{-/-} mice had increased levels of IL-5 and IL-6 but negligible amounts of IL-17 in BAL fluid; eosinophilic inflammation and AHR to inhaled methacholine were markedly reduced compared with those in WT mice. Transfer of iTreg cells into sensitized and challenged CD8^{-/-} recipients resulted in enhancement of lung allergic responses. Little IL-17 gene or protein expression was detected in these iTreg cells before transfer, and increased levels of IL-17 in BAL fluid were only observed in recipients of cells that were TCR activated together with TGF- β but not in recipients of cells after TCR activation alone or cultured with IL-6 (without TGF- β). These results identified the importance of TGF- β in staging the *in vivo* reprogramming and conversion of transferred iTreg cells. This staging was associated with reductions in *Foxp3* gene expression but higher *Ror γ t* and *Il17* mRNA levels, as demonstrated in transferred cells recovered from the lungs of sensitized and challenged CD8^{-/-} recipients.

The requirements for TGF- β and IL-6 are well established for expression of the lineage-specific transcription factor ROR γ t and *in vitro* differentiation of T_H17 cells.^{16-18,23} Furthermore, host IL-6 was essential for the transcriptional reprogramming of transferred iTreg cells, enabling conversion of these cells in recipients from suppressors to IL-17⁺ Teff cells and expression of ROR γ t.⁵ These findings were consistent with *in vitro* responses seen in CD4⁺CD25⁻ T cells exposed to TGF- β and IL-6 together (T_H17-polarizing conditions) or initially to TGF- β and then IL-6, in which increased expression of *Ror γ t* and *Il17* was detected. The importance of IL-6 in *in vitro* and *in vivo* differentiation of T_H17 cells was not surprising. IL-6 triggered phosphorylation of signal transducer and activator of transcription 3, which was required for induction of *Ror γ t* expression,¹⁸ promoting T_H17 cell differentiation.^{5,16,23} These events were seen in association with limited TGF- β -induced expression of *Foxp3*^{5,16} and attenuation of iTreg cell-mediated suppressor cell activity together with decreased *Foxp3* expression.⁵ Exposure of TCR-activated cells to IL-6 alone did not enhance lung allergic responses in sensitized and challenged CD8^{-/-} recipients and induced little *Ror γ t* gene expression or IL-17 mRNA and protein expression. When TGF- β was added 2 to 4 days after cells were exposed to IL-6, limited differentiation of T_H17 cells or expression of *Ror γ t* and IL-17 was detected. It is unclear at present what mechanisms underlie this IL-6-inducible refractoriness to TGF- β . In a number of IL-17-dependent immunopathologies, including diabetes⁹ and experimental autoimmune encephalomyelitis,^{16,23} the relative timing of TGF- β and IL-6 exposure appeared critical in determining disease outcomes.

TGF- β -induced expression of *Foxp3* was dependent on Smad3.^{20,24,25} Deletion of the Smad3 binding site in the CNS1 of the *Foxp3* promoter decreased expression of *Foxp3*.^{19,20} In keeping with these findings, we showed that TGF- β was critical to the induction process because cells treated with the Smad3 inhibitor during iTreg cell differentiation did not suppress development of lung allergic responses in sensitized and challenged WT recipients in association with decreased *Foxp3* gene expression and IL-10 gene and protein levels. In contrast, cells treated with the Smad3 inhibitor after TGF- β -induced differentiation maintained the suppressor phenotype in association with significant

expression of *Foxp3* and IL-10 and prevented development of experimental asthma.

TGF- β -mediated activities were shown to be dependent on distinct pathways, one dependent on Smad3 for development of suppressor function and the other dependent on TAK1 for generation of T_H17 cells and enhancement of lung allergic responses. In different cell types, activation of TAK1 can be triggered by diverse stimuli, including TGF- β , TNF- α , IL-1, and Toll-like receptors,²⁹ resulting in a broad spectrum of actions, including embryogenesis,³³ generation of CD4⁺ and CD8⁺ thymocytes, and development of CD4⁺CD25⁺ regulatory T cells.³⁴⁻³⁶ The results of kinetic experiments showed robust *Tak1* gene expression at early time points, as early as 6 hours after addition of TGF- β , suggesting that TAK1 might be relevant during the initial stages of differentiation. Expression of *Tak1*, similar to expression of *Foxp3*, was TGF- β dose dependent but not dependent on IL-6. Increasing *Tak1* mRNA expression preceded ROR γ t gene and protein expression and production of IL-17 in cells exposed to both TGF- β and IL-6; TCR-activated cells in the presence of TGF- β alone did not express ROR γ t or IL-17, despite upregulation of TAK1 gene and protein levels. These results suggest that signaling through TGF- β /TAK1 led to poising of the *Ror γ t* locus for optimal IL-6-mediated transcription, translocation, and differentiation of T_H17 cells.

Further highlighting this molecular priming effect, cells exposed to TGF- β followed by IL-6 exhibited robust *Ror γ t* expression, whereas cells subjected to the reverse order of addition were mainly refractory and exhibited lower *Ror γ t* mRNA expression levels. One outcome of TGF- β signaling in the presence of IL-6 was facilitation of transcription at the *Ror γ t* locus, as suggested by enrichment of H3K4m3 in association with decreases in H3K27m3 levels. This is consistent with reported opposing H3K4m3 and H3K27m3 modifications associated with gene activation and repression, respectively,^{37,38} and the activity of the H3K27 demethylase JMJD3 was critical to T_H17 cell differentiation.³⁹ The *Foxp3* locus exhibited nominal changes in trimethylation of histone proteins after TGF- β -dependent suppressor cell differentiation. This is in keeping with other potential epigenetic or regulatory mechanisms that affect *Foxp3* expression, including H3 acetylation,⁴⁰ DNA methylation,⁴¹ H3K4 dimethylation and trimethylation at early time points in cell development,⁴² and specific transcription factors.⁴³

Significantly, TAK1 was essential to the development of T_H17 cells and iTreg cell reprogramming into IL-17⁺ Teff cells. Inhibition of TAK1 using either a TAK1-specific inhibitor or shRNA prevented cells differentiated under T_H17-polarizing conditions (TGF- β and IL-6) from producing IL-17 or expressing *Ror γ t*. Furthermore, the loss of TAK1 in iTreg cells abolished iTreg cell-mediated enhancement of lung allergic responses in CD8^{-/-} recipients by preventing *in vivo* conversion, as demonstrated by negligible levels of expression of *Ror γ t* or *Il17* in recovered iTreg cells from the lungs of recipients. However, disruption of TAK1 activity did not interfere with iTreg cell suppressor function because transfer of these treated cells was effective in preventing the development of AHR and airway inflammation and decreases in levels of BAL fluid IL-13 in sensitized and challenged WT recipients. Thus blocking TGF- β signaling through TAK1 prevented iTreg cell to T_H17 cell reprogramming but left intact the TGF- β -Smad3 cascade for iTreg cell suppressor function. Importantly, colocalization of both Smad3 and TAK1 in the same TGF- β -treated cell further corroborated the dichotomous role of TGF- β and its seemingly

paradoxical involvement in divergent developmental pathways and generation of T suppressor or T_H17 cells.

In summary, the data demonstrated that TGF- β regulates the development, commitment, and cell fate of iTreg and T_H17 cells through different signal transduction pathways, one Smad3 dependent and the other TAK1 dependent. It appears that in the presence of IL-6, the TGF- β -dependent suppressor pathway is overridden by one leading to T_H17 differentiation. Exposure of TCR-activated cells to TGF- β appeared to poise cell susceptibility to IL-6 in a TAK1-dependent manner, in part through histone modifications at the *Ror γ t* locus. The ability to modulate inducible regulatory T-cell function and balance the iTreg cell-T_H17 axis through manipulation of the activities of Smad3 or TAK1 has clinical implications for the treatment of immunopathologies associated with IL-17-mediated autoimmunity and allergy.

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Key messages

- Inducible regulatory T cells exhibit capacity for transcriptional reprogramming, converting from suppressor cells of lung allergic responses to enhancing IL-17-producing effector cells when activated by IL-6.
- Because TGF- β is essential to both of these pathways, maintaining a balance is critical to prevent neutrophil-mediated autoimmune disease or neutrophil-dependent asthma.

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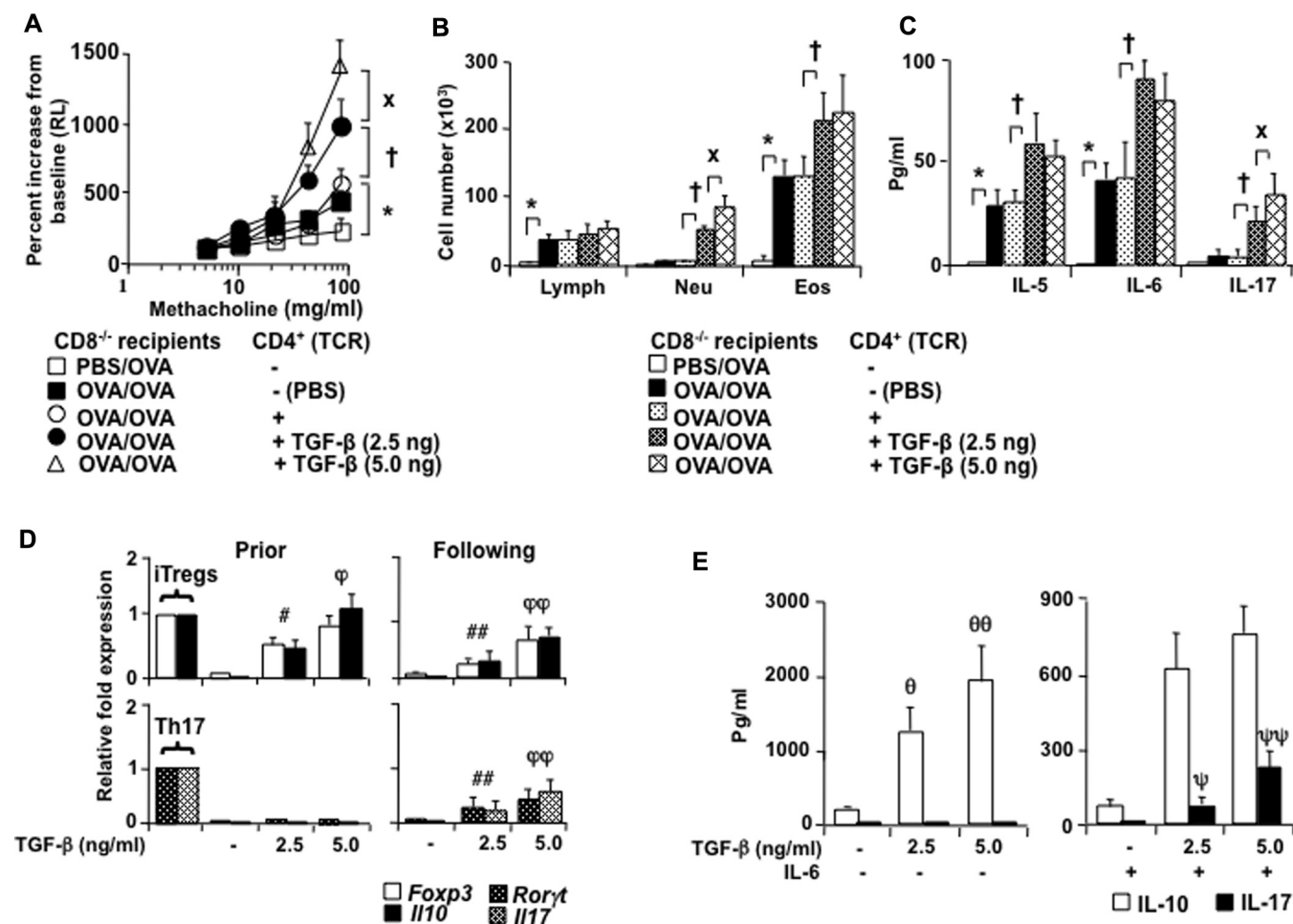


FIG E1. TGF- β regulates iTreg cell-mediated enhancement of lung allergic responses in sensitized and challenged CD8^{-/-} recipients and gene expression and *in vitro* cytokine production in a dose-dependent manner. **A**, AHR. **B**, BAL fluid cell composition. *Eos*, Eosinophils; *Lymph*, lymphocytes; *Neu*, neutrophils. **C**, BAL fluid cytokine levels. **D**, Gene expression in cells before and after transfer and recovery from lungs of sensitized and challenged CD8^{-/-} recipients. **E**, Cytokine production. Shown are means $\pm$ SEMs from 3 independent experiments (4 mice per group, $n = 12$). * $P < .05$ comparing sensitized and challenged CD8^{-/-} mice with mice challenged only. † $P < .05$ comparing recipients of cells activated by TCR alone with cells exposed to low-dose TGF- β . X $P < .05$ comparing recipients of cells exposed to high-dose (5 ng/mL) versus low-dose (2.5 ng/mL) TGF- β . #Cells before transfer and ##recovered cells: $P < .05$ comparing cells exposed to low-dose TGF- β (2.5 ng/mL) to TCR activation alone. ϕ Cells before transfer and $\phi\phi$ recovered cells: $P < .05$ comparing cells exposed to high-dose versus low-dose TGF- β (taking gene expression of iTreg or Th17 cells as 1). θ IL-10 and ψ IL-17: $P < .05$ comparing cells exposed to low-dose TGF- β to TCR activation alone. $\theta\theta$ IL-10 and $\psi\psi$ IL-17: $P < .05$ comparing cells exposed to high-dose versus low-dose TGF- β .

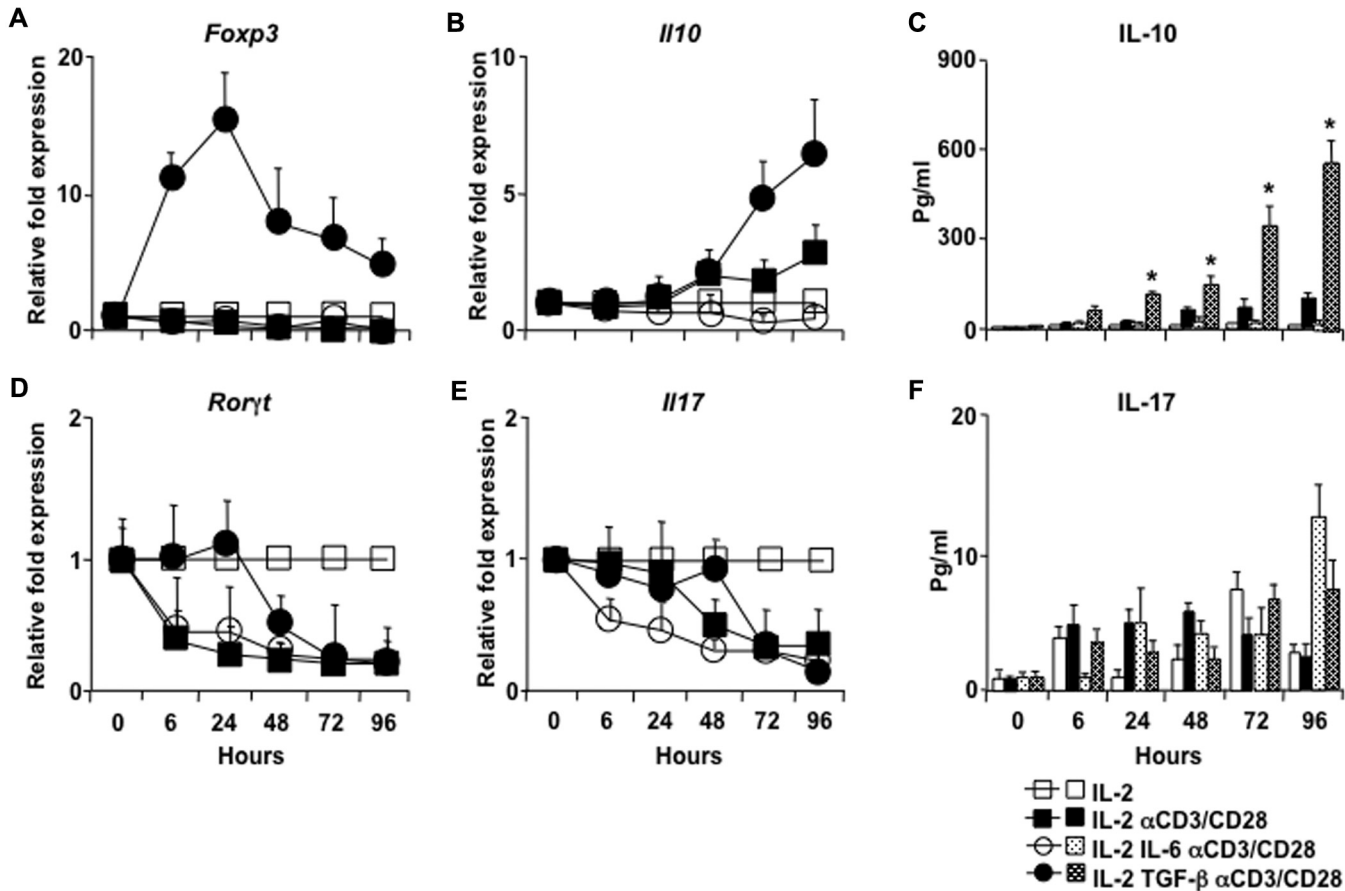


FIG E2. TGF- β regulates the kinetics of gene expression and *in vitro* production of cytokines. Quantitative gene (A, *Foxp3*; B, *Il10*; D, *Roryt*; E, *Il17*) expression levels of cells under different culture conditions over the 7-day time course are shown. *In vitro* production of cytokines (C, IL-10; F, IL-17) at each corresponding time point are also shown.

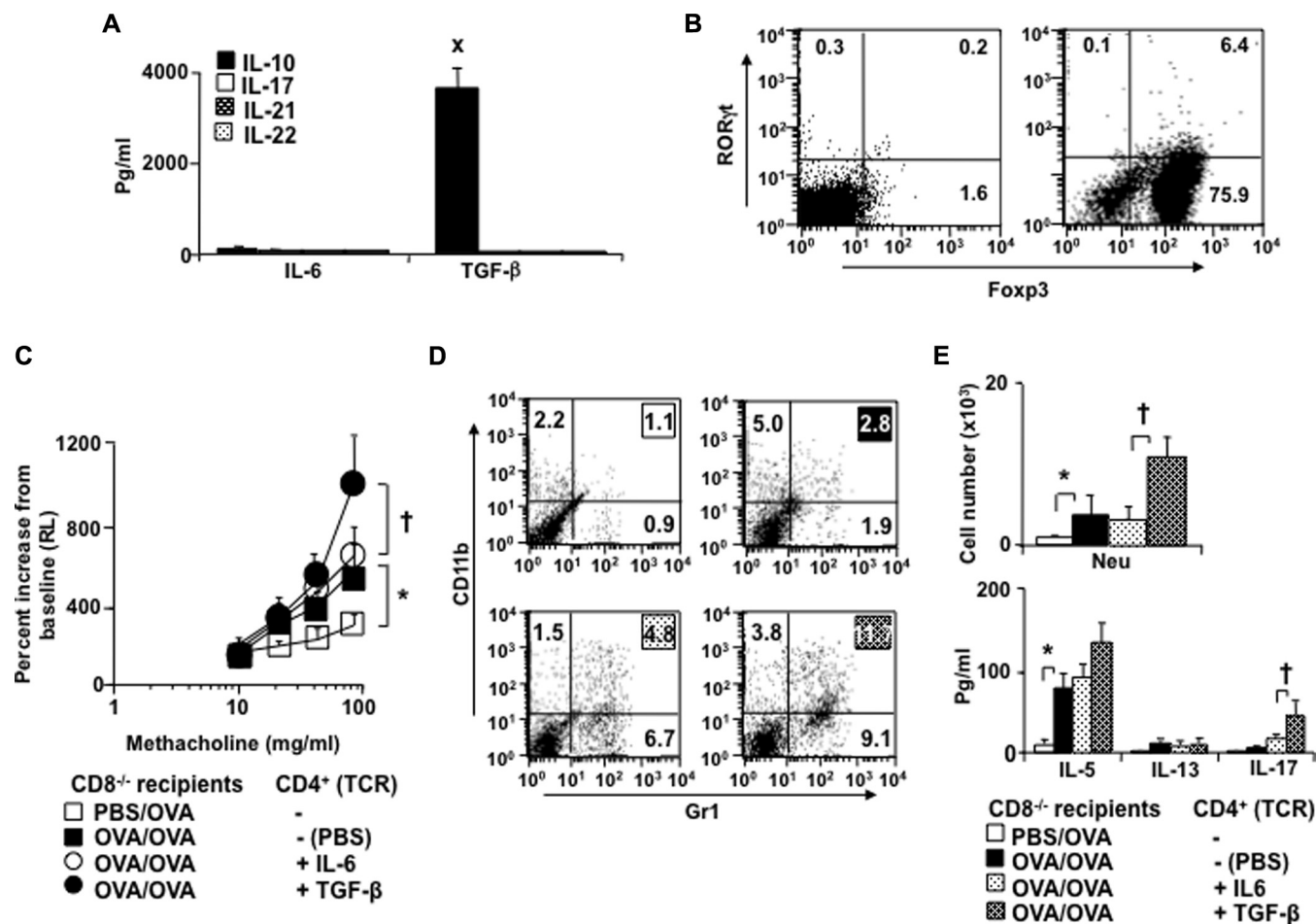


FIG E3. Cells exposed to TGF- β but not IL-6 enhance lung allergic responses in sensitized and challenged CD8^{-/-} recipients. **A**, *In vitro* cytokine production. **B**, Intracellular staining of proteins using flow cytometry. **C**, AHR. **D**, Flow cytometry. **E**, Cell morphology: BAL fluid neutrophils. **F**, BAL fluid cytokine levels. Shown are means $\pm$ SEMs from 3 independent experiments (4 mice per group, $n = 12$). $XP < .05$ comparing cells exposed to TGF- β or IL-6. $*P < .05$ comparing sensitized and challenged recipients of TCR-activated CD4⁺ T cells to mice challenged only. $\dagger P < .05$ comparing recipients of TCR-activated CD4⁺ T cells exposed to TGF- β or IL-6.

TABLE E1. Primer sequence for chromatin immunoprecipitation quantitative PCR experiments located in the *Roryt* promoter

Primer	Sequence (5'3')
<i>Roryt</i> _1_fwd	GTGTAGGAGGAATTTGGGTGTGG
<i>Roryt</i> _1_rev	GCAGTGGAGGTGGGGTGAAG
<i>Roryt</i> _2_fwd	CACACCAACAAAACCTGAAGAGAAAAC
<i>Roryt</i> _2_rev	CCACACCCAAATTCCTCCTACAC
<i>Roryt</i> _3_fwd	GGGAGGAAAGCGAGTCAGAATG
<i>Roryt</i> _3_rev	TCAGGTTTGCATTGCGAGTGTGTC
<i>Roryt</i> _4_fwd	AGAATGAGTGGACAGGAACCCG
<i>Roryt</i> _4_rev	CATTCTGACTCGCTTTCCTCCC