

Coexpression of TGF- β 1 and IL-10 Enables Regulatory T Cells to Completely Suppress Airway Hyperreactivity¹

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In allergic airway disease, Treg may play an important role in the modulation of airway hyperreactivity (AHR) and inflammation. We therefore investigated the therapeutic potential of Treg in an Ag-dependent murine asthma model. We here describe that AHR can be completely suppressed by adoptive transfer of Treg overexpressing active TGF- β 1. Using mice with impaired TGF- β signaling in T cells, we could demonstrate that TGF- β signaling in recipient effector T cells or transferred Treg themselves is not required for the protective effects on AHR. However, the expression of IL-10 by Treg was found to be essential for the suppression of AHR, since Treg overexpressing active TGF- β 1 but deficient in IL-10 lacked protective effects. Airway inflammation could not be significantly suppressed by wild-type or transgenic Treg. In conclusion, modulation of cytokine expression by Treg may have therapeutic potential for the treatment of AHR in asthma. The mechanisms of the effects of Treg on airway inflammation require further clarification. *The Journal of Immunology*, 2008, 181: 7751–7758.

The CD4⁺CD25⁺ T cells (Treg)³ are required to maintain immune homeostasis (1–3). In allergic airway disease, Treg may play an important role in the modulation of airway hyperreactivity (AHR) and inflammation (4–7). However, conflicting results have been reported on the ability of Treg to modulate AHR and/or inflammation. In particular, the relative contribution of TGF- β 1 and IL-10 in Treg control of AHR is insufficiently understood. Transfer of Ag-dependent Treg derived from lung down-regulated eosinophilic airway inflammation, but had no effect on AHR (4). However, it was also shown that transfer of Ag-dependent Treg was able to provide IL-10-dependent protection from AHR in the mouse (6).

The immunosuppressive cytokines TGF- β 1 and IL-10 are involved in the regulation of airway reactivity. In asthma patients, high levels of TGF- β have been found in bronchoalveolar lavage fluid (BALF) (8, 9). In mouse models, impairment of TGF- β signaling in T cells has led to an exacerbation of the asthmatic phenotype (10, 11). In addition, retroviral transduction of unselected CD4⁺ T cells with latent TGF- β 1 was shown to improve AHR and airway inflammation upon adoptive transfer (12). Data on the sus-

ceptibility of IL-10^{-/-} mice toward the induction of asthma are more controversial; both improvement and deterioration of allergic airway disease have been reported (5, 13, 14).

Since Treg have been shown to require IL-10 as well as TGF- β for full immunosuppressive function in vivo (15–18), we aimed to dissect the specific contributions of TGF- β 1 and IL-10 to the immunomodulatory capacity of Treg in a murine asthma model.

Asthma is characterized by AHR, airway inflammation, and increased mucus secretion (19). Th2 cytokines such as IL-4, IL-5, IL-10, and IL-13 seem to play a role in the pathogenesis of asthma (20, 21), but the mechanisms of AHR and pulmonary inflammation are incompletely understood and data on the role of specific cytokines are conflicting: IL-5-transgenic mice show marked tissue eosinophilia but do not demonstrate increased AHR (22). IL-13 appears to promote AHR and has been reported to variably affect pulmonary inflammation (23, 24). Therefore, AHR and airway inflammation seem to be differentially regulated in the pathogenesis of asthma.

We here report that Treg overexpressing active TGF- β 1 are able to confer complete resistance toward the induction of Ag-dependent AHR. This does not seem to depend on the direct suppression of effector T cells or the direct modulation of Treg via TGF- β signaling. However, expression of IL-10 by Treg was required for the suppression of AHR. Modulation of cytokine expression by Treg may therefore have therapeutic potential for the treatment of AHR in asthma.

Materials and Methods

Animals

Generation and characterization of the transgenic mouse lines hCD2-TGF- β 1 and hCD2-dkT β R11 have been previously described (25, 26). Transgenic lines were established and maintained as heterozygotes on a FVB/N or C57/B6 background. FVB/N or C57/B6 nontransgenic littermates were used as controls. All animals used in the experiments were age and sex matched and used between 6 and 8 wk of age. Animal care was in accordance with the governmental and institutional guidelines and all experiments were approved by the local review board.

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³ Abbreviations used in this paper: Treg, regulatory T cell; AHR, airway hyperreactivity; BALF, bronchoalveolar lavage fluid; MCh, methacholine; dTG, double transgenic; n.s., nonsignificant; RI, lung resistance; EF, expiratory flow; ASM, airway smooth muscle cell.

Sensitization and measurement of airway reactivity

Sensitization of mice as well as the measurement of airway reactivity in a head-out body plethysmograph were performed as previously described (11). The methacholine (MCh) dose resulting in a 50% decrease of the tidal mid-expiratory flow (EF₅₀), a marker for airway resistance, was measured. In brief, animals were immunized with 100 μ g of OVA-alum or PBS-alum on days 0 and 14 i. p. On days 25–27, mice were aerosol challenged with 1% OVA in PBS for 20 min. AHR was measured on day 28 in untreated (PBS-alum) and sensitized (OVA-alum) wild-type (WT), hCD2-TGF- β 1-transgenic, hCD2-TGF- β 1 $\times$ hCD2-dkT β RII double-transgenic (dTG), and IL-10^{-/-} mice as well as in sensitized WT mice or hCD2-dkT β RII-transgenic mice that had adoptively received 3.5×10^5 CD4⁺CD25⁺ or CD4⁺CD25⁻ T cells from either WT, hCD2-TGF- β 1, hCD2-TGF- β 1 $\times$ hCD2-dkT β RII, or hCD2-TGF- β 1 $\times$ IL-10^{-/-} animals. Adoptive transfer was performed at least 3 h before the first aerosol challenge. To suppress IL-10 production of adoptively transferred hCD2-TGF- β 1 CD4⁺CD25⁺ cells in IL-10^{-/-} mice, 62.5 μ g of a monoclonal, neutralizing anti IL-10 Ab clone JES052A5 or rat IgG1 isotype control clone 43414 (both R&D Systems) were injected i.p. on days 25 and 27 (Fig. 1).

To confirm the validity of data generated by head-out plethysmography, invasive body plethysmography was performed in WT and hCD2-TGF- β 1-transgenic mice (27). Invasive plethysmography was then used for some experiments with units for lung resistance (RI) given as indicated. In brief, dose response was assessed in anesthetized mice by i.v. administration of increasing amounts of MCh (33–1000 μ g/kg) (27).

Cell isolation

For the isolation of CD4⁺CD25⁺ cells, MACS beads (Miltenyi Biotec) were used to positively select CD25⁺ cells. These cells were then sorted for CD4 with a FACSVantage cell sorter (BD Biosciences). The flow-through of the CD25⁺ selection was used to positively select CD4⁺CD25⁻ cells with MACS beads. The purity of cell isolation with regard to CD25 expression ranged between 96 and 99% and >90% of CD25⁺ cells expressed Foxp3 as assessed by flow cytometry (Fig. 2). Donor mice used for cell isolation were immunized with OVA-alum i.p. on days 0 and 14. Unless indicated otherwise, 3.5×10^5 cells were transferred in all experiments.

Flow cytometry

Flow cytometric analysis was performed using a FACSCanto (BD Biosciences) along with FACSDiva software. At least 1×10^6 cells were analyzed. The following Abs were used: CD4-PE, CD25-FITC (BD Biosciences), Foxp3-allophycocyanin (eBioscience, Frankfurt, Germany).

Collection of BALF and lung preparation

After AHR measurement, BALF was collected as previously described (11). Cells were counted with a CASY (Schaerfe System). One hundred microliters of the cell suspension was used for cytospin preparations that were stained with a Diff-Quick staining kit (Medion Diagnostics).

Cytokine detection

IL-13 concentrations in BALF were measured using the Mouse Quantikine ELISA kit (R&D Systems) according to the manufacturer's instructions. For the detection of active TGF- β 1 in BALF, the TGF- β mouse Duo Set kit by R&D Systems was used. Levels in BALF were measured after acid activation with H₂SO₄.

Th1/Th2 cytokines in BALF were analyzed using the FlowCytomix Multiplex mouse Th1/Th2 kit from Bender MedSystems according to the manufacturer's instructions.

Histological assessment of inflammation

The lungs were inflated and fixed in formalin and embedded in paraffin. Five-micrometer sections were stained with H&E. For the determination of the degree of histological inflammation, H&E-stained lung sections were scanned with a digital camera (Olympus) and the areas of inflammation were measured using analy-SIS 3.0 software (Software Imaging Systems). The percentage of inflamed area in relation to the lung area scanned is given. Three representative areas per lung were chosen for measurement.

Statistical analysis

For comparison of groups, the Mann-Whitney *U* test was used. Mean and SEM are given and a *p* < 0.05 was considered significant.

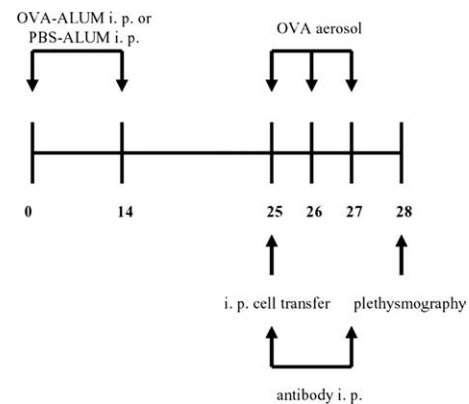


FIGURE 1. Schematic immunization protocol. Animals were immunized with OVA-alum or PBS-alum on days 0 and 14. On days 25–27, mice were aerosol challenged with 1% OVA in PBS for 20 min. In some experiments, 62.5 μ g of neutralizing anti-IL-10 Ab or isotype control was given on days 25 and 27 three hours before aerosol challenge. On day 28, plethysmography was performed. The MCh dose that results in a 50% decrease of the tidal mid-EF₅₀ is given, unless otherwise indicated.

Results

Treg, but not CD4⁺CD25⁻ T cells overexpressing active TGF- β 1 are potent suppressors of Ag-specific AHR

The first aim of our study was to assess Treg as potential modulators of AHR, a hallmark of asthma. We therefore determined whether transfer of naturally occurring Treg could prevent the development of AHR. Treg (3.5×10^5) isolated from OVA-immunized WT mice were injected i.p. into OVA-immunized WT recipient mice 3 h before the first OVA aerosol challenge (Fig. 1). To confirm the phenotype of transferred cells, flow cytometric analysis of isolated cells was performed and CD25⁺ T cells were shown to express Foxp3 in over 90% (Fig. 2). Immunized WT mice showed a strong Ag-dependent AHR upon OVA aerosol challenge (MCh₅₀, WT-OVA: 26.3 ± 2.1 mg/ml vs WT-PBS: 54.23 ± 4.3 mg/ml, *p* = 0.0006; Fig. 3A). Transfer of 3.5×10^5 WT Treg did not protect from Ag-dependent AHR upon adoptive transfer (MCh₅₀, WT-OVA: 26.3 ± 2.1 mg/ml vs WT-OVA plus WT Treg: 16.99 ± 1.4 mg/ml, *p* = 0.006; Fig. 3A). Transfer of WT CD4⁺CD25⁻ T cells did not alter airway reactivity

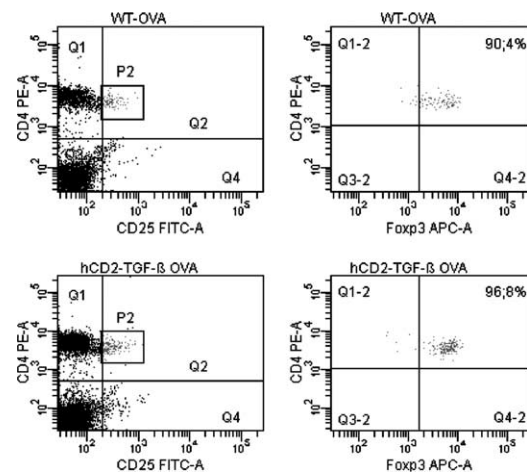
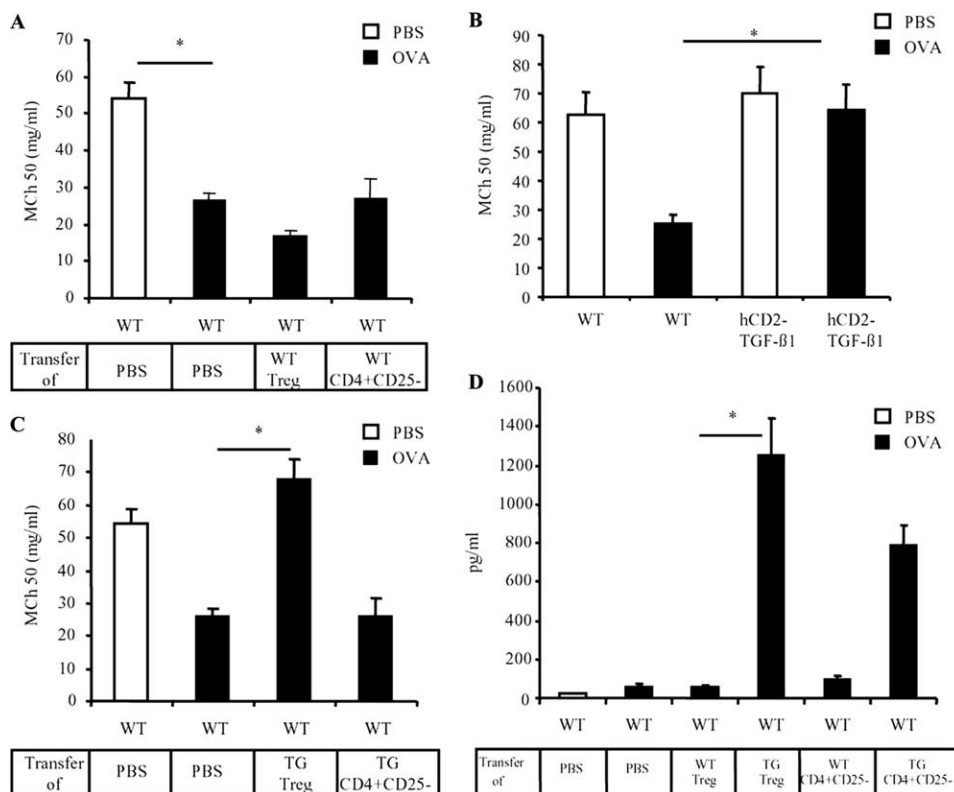


FIGURE 2. Flow cytometric analysis of WT and hCD2-TGF- β 1 Treg. Cells were isolated from spleen of OVA-immunized mice and stained with anti CD4-PE, anti CD25-FITC, and anti Foxp3-allophycocyanin AB. Cells (1×10^6) were analyzed showing that >90% of WT and transgenic CD4⁺CD25⁺ T cells were positive for Foxp3.

FIGURE 3. Adoptive transfer of Treg overexpressing active TGF- β 1 leads to protection from AHR. The schematic representation of the experimental setting is given in Fig. 1. Adoptive transfer of cells was performed on day 25 at least 3 h before the first aerosol challenge. **A**, Adoptive transfer of 3.5×10^5 WT Treg or CD4 $^+$ CD25 $^-$ T cells in WT recipient mice. **B**, AHR in WT and hCD2-TGF- β 1-transgenic mice. **C**, Adoptive transfer of 3.5×10^5 transgenic hCD2-TGF- β 1 Treg or the same number of transgenic CD4 $^+$ CD25 $^-$ T cells into WT recipient mice. **D**, BALF levels of active TGF- β 1 were determined using ELISA. These experiments included six to eight mice per group and were repeated twice with similar results (*, $p = 0.001$).



(MCh₅₀, WT-OVA: 26.3 ± 2.1 mg/ml vs WT-OVA+WT CD4 $^+$ CD25 $^-$: 26.95 ± 5.2 mg/ml, nonsignificant (n.s.); Fig. 3A). By increasing the number of transferred WT-Treg to 5×10^5 and 1×10^6 cells, we could observe a nonsignificant decrease in AHR (data not shown). We can therefore not exclude that higher doses of WT-Treg may provide protection from Ag-induced AHR.

We next investigated whether the expression of regulatory cytokines such as TGF- β 1 may enhance the suppressive effects of Treg on AHR. TGF- β 1 has been previously shown to modulate airway reactivity and inflammation (11, 12, 28). Therefore, we were interested whether overexpression of TGF- β 1 may increase the therapeutic potential of Treg in asthma. Treg were isolated from transgenic mice with T cells overexpressing a mutated and thus constitutively active form of TGF- β 1 which had been previously generated in our laboratory (hCD2-TGF- β 1; Ref. 26). These mice were resistant to the induction of Ag-dependent AHR (MCh₅₀ WT-OVA: 25.34 ± 8.35 mg/ml vs hCD2-TGF- β 1-OVA: 64.24 ± 8.65 mg/ml, $p = 0.003$; Fig. 3B). Upon adoptive transfer, 3.5×10^5 hCD2-TGF- β 1 Treg were able to completely protect from AHR (MCh₅₀ WT-OVA: 26.3 ± 2.1 mg/ml vs WT-OVA+hCD2-TGF- β 1 Treg: 67.9 ± 6.2 mg/ml, $p = 0.001$; Fig. 3C). Mice receiving hCD2-TGF- β 1 Treg even tended to be less susceptible to develop airway constriction to stimulation with MCh than sham-immunized mice (MCh₅₀ WT-PBS: 54.23 ± 4.3 mg/ml vs WT-OVA plus hCD2-TGF- β 1 Treg: 67.9 ± 6.2 mg/ml, n. s.; Fig. 3C).

In contrast, hCD2-TGF- β 1 CD4 $^+$ CD25 $^-$ T cells were not able to transfer any protection from AHR, suggesting that TGF- β 1 acts in the context of Treg and does not protect from AHR on its own (MCh₅₀ WT-OVA: 26.3 ± 2.1 mg/ml vs WT-OVA plus hCD2-TGF- β 1 CD4 $^+$ CD25 $^-$: 25.9 ± 5.5 mg/ml, n. s.; Fig. 3C). Accordingly, we could demonstrate that levels of TGF- β 1 in BALF did not correlate with protection from AHR: the levels of TGF- β 1 in BALF of WT mice that were protected from AHR by receiving hCD2-TGF- β 1 Treg were not significantly different than the levels

of mice not protected from AHR and having received hCD2-TGF- β 1 CD4 $^+$ CD25 $^-$ T cells (WT-OVA plus hCD2-TGF- β 1 Treg: 1254.6 ± 191.5 pg/ml vs WT-OVA plus hCD2-TGF- β 1 CD4 $^+$ CD25 $^-$: 791.1 ± 99.3 pg/ml, n. s.; Fig. 3D). Transfer of Tregs isolated from hCD2-TGF- β 1-transgenic mice resulted in markedly increased TGF- β 1 BALF levels compared with mice transferred with Treg from WT mice which showed levels similar to untransferred WT animals (WT-OVA plus hCD2-TGF- β 1 Treg: 1254.6 ± 191.5 pg/ml vs WT-OVA plus WT Treg: 56.91 ± 9.82 pg/ml, $p = 0.0007$; Fig. 3D). Therefore, the context of TGF- β 1 production rather than the amount of cytokine seems to determine protection from AHR.

TGF- β signaling in recipient T cells and in transferred Treg themselves is not required for the protection from AHR

To analyze the mechanisms involved in TGF- β 1-mediated suppression of AHR, hCD2-TGF- β 1 mice were crossed with transgenic mice with an impaired TGF- β signaling in T cells due to overexpression of a dominant negative TGF- β type II receptor (hCD2-dkT β RII (25)).

In contrast to single-transgenic hCD2-TGF- β 1 mice, OVA-immunized double-transgenic animals (dTG-OVA) were not protected from AHR and demonstrated airway responses similar to those of WT mice (MCh₅₀, WT-OVA: 25.34 ± 8.35 mg/ml vs dTG-OVA: 29.6 ± 10.9 mg/ml, n.s.; Fig. 4A). We have previously demonstrated that hCD2-dkT β RII-OVA have similar MCh₅₀ as WT-OVA animals (11). To dissect the effects of TGF- β 1 on recipient T cells or transferred Treg from other mechanisms involved, Treg from dTG mice were isolated overexpressing active TGF- β 1 and with an impaired TGF- β signaling pathway. These dTG Treg were then adoptively transferred into WT mice. In contrast to dTG mice, WT mice receiving dTG Treg were completely protected from AHR, indicating that TGF- β signaling in transferred Treg themselves is not required for protection from AHR (MCh₅₀, WT-OVA: 26.3 ± 2.1 mg/ml vs WT-OVA plus dTG

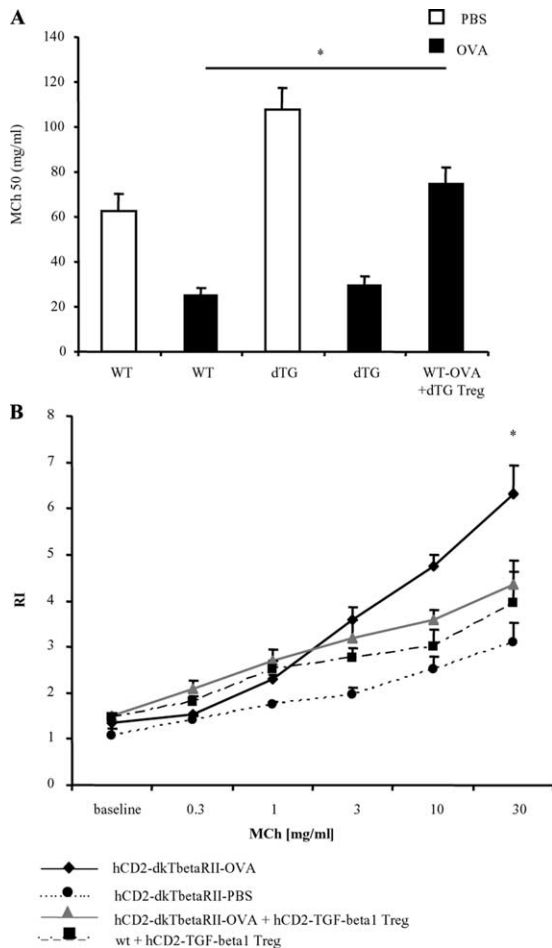


FIGURE 4. TGF- β signaling in recipient T cells or transferred Treg is not required for the protection from AHR. Adoptive transfer of cells was performed on day 25 at least 3 h before the first aerosol challenge. **A**, AHR in WT and dTG hCD2-TGF- β 1 $\times$ hCD2- Δ KT β RII mice and in WT mice receiving dTG Treg (*, $p = 0.001$). **B**, Invasive body plethysmography was performed in hCD2- Δ KT β RII mice receiving hCD2-TGF- β 1-transgenic Treg. Values for RI are given (*, $p = 0.06$). These experiments included four to eight mice per group.

Treg: 74.6 ± 7.4 mg/ml, $p = 0.001$; Fig. 4A). To assess the requirement of TGF- β signaling in recipient effector T cells, TGF- β 1 overexpressing Treg were transferred into mice with an impaired TGF- β signaling pathway in T cells. Again, these animals were protected from AHR, indicating that TGF- β 1 produced by transferred Treg may not directly act on recipient T cells (RI as measured by invasive body plethysmography at a dose of 30 mg/ml MCh: hCD2-dkT β RII-OVA: 6.31 ± 0.6 vs hCD2-dkT β RII-OVA plus hCD2-TGF- β 1 Treg: 4.34 ± 0.5 , $p = 0.06$; Fig. 4B). TGF- β signaling in T cells therefore seems to be required for the TGF- β 1-induced protection from AHR in transgenic animals. However, the suppressive capacity of transferred Treg does not seem to depend on TGF- β signaling in Treg themselves or in recipient T cells. Since this difference cannot be explained by the effects of TGF- β alone, we next examined the role of other suppressive cytokines which may be involved in Treg-induced protection from AHR.

Expression of IL-10 is necessary for the protective effects of Treg overexpressing TGF- β 1

IL-10 as well as TGF- β 1 have been involved in the effects of Treg on asthma (5, 11, 28). We have previously shown that overexpres-

sion of TGF- β 1 in T cells from hCD2-TGF- β 1-transgenic mice induces the expression of IL-10 within these cells (26). Therefore, we next aimed to dissect the relative contributions of IL-10 and TGF- β 1 to the observed protection from AHR by transgenic Treg. To this end, hCD2-TGF- β 1 Treg as well as WT Treg were transferred into IL-10-deficient mice (IL-10 $^{-/-}$). To eliminate IL-10 produced by transferred cells themselves, neutralizing anti-IL-10 Ab was injected in addition.

IL-10 $^{-/-}$ mice were significantly less susceptible to AHR than WT animals (MCh₅₀, WT-OVA: 26.3 ± 2.1 mg/ml vs IL-10 $^{-/-}$ OVA: 67.85 ± 6.75 mg/ml, $p = 0.004$; Fig. 5A), indicating that IL-10 is required for the induction of AHR, as was previously shown (13). Adoptive transfer of hCD2-TGF- β 1 Treg into IL-10 $^{-/-}$ mice further increased resistance to MCh-induced airway reactivity up to the level that even the highest concentrations of MCh used did not induce airway constriction (MCh₅₀, IL-10 $^{-/-}$ OVA: 67.85 ± 6.75 mg/ml vs IL-10 $^{-/-}$ OVA plus hCD2-TGF- β 1 Treg: 125 ± 0 mg/ml, $p = 0.01$; Fig. 5A). IL-10 $^{-/-}$ mice that received transgenic Treg along with neutralizing IL-10 Ab however showed significantly less protection from MCh-induced airway constriction (MCh₅₀, IL-10 $^{-/-}$ OVA plus hCD2-TGF- β 1 Treg plus AB: 80.5 ± 5.9 mg/ml, $p = 0.02$; Fig. 5A). WT Treg transferred with or without anti-IL-10 did not affect AHR in IL-10 $^{-/-}$ mice (MCh₅₀, IL-10 $^{-/-}$ OVA: 67.85 ± 6.75 mg/ml vs IL-10 $^{-/-}$ OVA + WT Treg: 60.1 ± 5.3 mg/ml vs IL-10 $^{-/-}$ OVA plus WT Treg plus AB: 54.73 ± 5.53 mg/ml, n.s.; Fig. 5B).

Since IL-10 $^{-/-}$ mice demonstrated weak Ag-dependent AHR, we used invasive body plethysmography to validate these data. On invasive body plethysmography, IL-10 $^{-/-}$ mice demonstrated Ag-dependent AHR, which was less pronounced as compared with WT mice, but which confirmed the relative resistance of IL-10 $^{-/-}$ mice toward MCh-induced airway reactivity (Fig. 5C).

These results suggested a dual role for IL-10 in the modulation of AHR; on the one hand, IL-10 seems to be required for the development of AHR, in contrast IL-10 strongly protects from AHR when coexpressed with TGF- β 1 on Treg. To further investigate this, hCD2-TGF- β 1 mice were crossed onto the IL-10 $^{-/-}$ background to obtain T cells that overexpress TGF- β 1 but are deficient in IL-10. Treg from hCD2-TGF- β 1 $\times$ IL-10 $^{-/-}$ mice were then transferred into WT animals. Indeed, Treg overexpressing TGF- β 1 but deficient in IL-10 completely lost their protective effects on AHR (MCh₅₀, WT-OVA: 26.3 ± 2.1 mg/ml vs WT-OVA plus IL-10 $^{-/-}$ $\times$ hCD2-TGF- β 1 Treg: 28.8 ± 6.8 mg/ml, n.s.; Fig. 5D).

Taken together, these data demonstrate that expression of IL-10 is essential for the protective effects mediated by Treg overexpressing active TGF- β 1.

WT Treg reduce airway eosinophilia but not airway inflammation

AHR and airway inflammation often coincide. However, increasing evidence suggests that these two may not be directly related with regard to pathophysiological mechanisms (13, 22, 23). We therefore investigated the suppressive effects of Treg on airway inflammation as well. Adoptive transfer of 3.5×10^5 WT Treg into WT mice which were not protected from AHR led to a marked decrease in BALF eosinophil numbers (WT-OVA: $0.27 \times 10^6 \pm 0.05 \times 10^6$ cells vs WT-OVA plus WT Treg: $0.047 \times 10^6 \pm 0.017 \times 10^6$ cells, $p = 0.038$; Fig. 6A), but did not significantly affect the total area of inflamed lung tissue (WT-OVA: $10.1 \pm 0.57\%$ vs WT-OVA plus WT Treg: $10.11 \pm 1.14\%$; Fig. 6B). Transfer of WT CD4 $^{+}$ CD25 $^{+}$ T cells did not alter BALF eosinophil count or airway inflammation (Fig. 6A).

FIGURE 5. Expression of IL-10 is required for the protective effects of hCD2-TGF- β 1 Treg on AHR. **A**, AHR in IL-10 $^{-/-}$ mice receiving transgenic hCD2-TGF- β 1 Treg with or without neutralizing anti-IL-10 Ab (*, $p = 0.01$; **, $p = 0.02$). **B**, AHR in IL-10 $^{-/-}$ mice receiving WT Treg with or without neutralizing anti-IL-10 Ab. **C**, Invasive body plethysmography in IL-10 $^{-/-}$ mice receiving transgenic hCD2-TGF- β 1 Treg with neutralizing anti-IL-10 Ab or IgG. **D**, AHR in WT mice receiving transgenic hCD2-TGF- β 1 Treg or transgenic hCD2-TGF- β 1 Treg deficient in IL-10 (*, n.s.). These experiments included six to eight mice per group and were repeated twice with similar results.

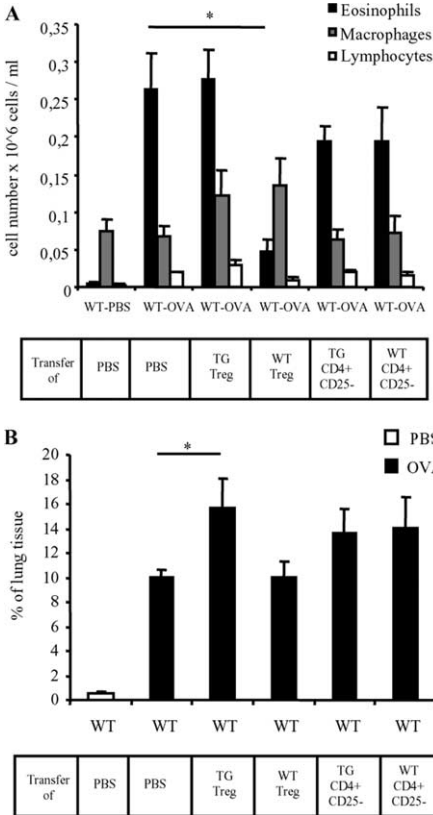
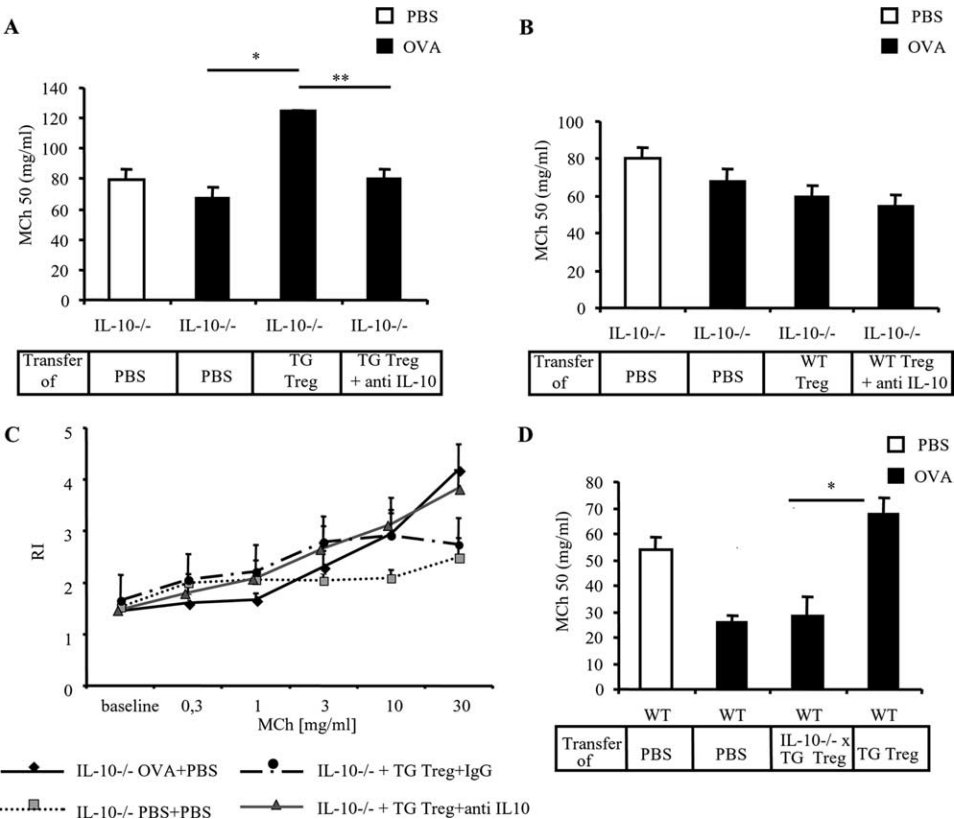


FIGURE 6. Effects of adoptively transferred Treg on airway inflammation. **A**, Analysis of BALF cell numbers. Lungs were lavaged after the assessment of AHR on day 28 and recovered cells were assessed using cytopsin preparations ($p = 0.04$). **B**, Analysis of inflamed lung tissue. Removed lungs were fixed in formalin and 5- μ m-thick sections were stained with H&E. Areas of inflammation are given in relation to the whole lung area ($p = 0.04$). Six to eight mice per group were analyzed.

Overexpression of active TGF- β 1 was not able to enhance the suppressive effects of WT Treg on airway inflammation (eosinophil count: WT-OVA: $0.27 \times 10^6 \pm 0.05 \times 10^6$ cells vs WT-OVA+hCD2-TGF- β 1 Treg: $0.28 \times 10^6 \pm 0.04 \times 10^6$ cells; Fig. 6A, and area of inflamed lung tissue: WT-OVA: $10.1 \pm 0.57\%$ vs WT-OVA plus hCD2-TGF- β 1 Treg: $15.71 \pm 2.33\%$, $p = 0.04$; Figs. 6B and 7). In contrast, these cytokines may even enhance airway inflammation despite the protective effects observed on AHR. These results suggest separate mechanisms regulating airway reactivity and inflammation.

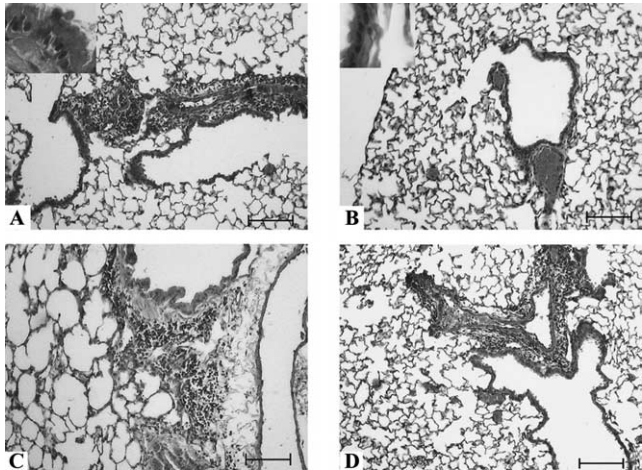


FIGURE 7. Representative histological sections of lung tissue. **A**, OVA-immunized WT animals with peribronchial inflammation and goblet cell hyperplasia. **B**, Sham-immunized WT mice without airway inflammation. **C**, Airway inflammation in WT mice adoptively transferred with hCD2-TGF- β 1 Treg. **D**, Airway inflammation in WT mice adoptively transferred with WT Treg. Bars, 100 μ m.

Cytokine levels in BALF

IL-13 may play a critical role in the regulation of asthma (29). However, we found no correlation between protection from AHR and levels of IL-13 in BALF after adoptive cell transfer (data not shown). BALF levels of IL-5 were found to correlate with BALF eosinophilia, corresponding to its role in eosinophil migration (data not shown). All other cytokines tested, including IL-1, IL-2, IL-4, IL-6, IL-10, IL-17, TNF- α , IFN- γ , and GM-CSF, were either not detectable in BALF or showed no correlation with the development of AHR or inflammation (data not shown).

Discussion

Immunotherapy of allergic diseases aims at specific treatment associated with minimal side effects. We were therefore interested in the modulation of airway responses by Treg in a murine asthma model. We here demonstrate that relatively low numbers of adoptively transferred Treg overexpressing active TGF- β 1 completely protected mice from the induction of Ag-dependent AHR. This effect did not depend on an intact TGF- β signaling pathway within recipient T cells or transferred Treg but on the expression of IL-10.

Previous studies have suggested that naturally occurring Treg may have an effect on airway inflammation but not on AHR in murine models of asthma (4, 30). However, data are conflicting and, using cell depletion, it has been shown that CD25⁺ T cells may affect both AHR and airway inflammation (31). Upon adoptive transfer of 3.5×10^5 WT Treg, we were not able to demonstrate a suppressive effect on AHR and the effect could not be significantly increased by increasing the number of cells transferred. These discrepant results may relate to different models and mouse strains used but suggest that naturally occurring Treg may not be suitable candidates for immunotherapy of asthma using reasonably low numbers of cells.

During the past few years, it has been shown that different subsets of cells with a regulatory phenotype may modulate airway responses (32, 33). We therefore investigated whether the regulatory cytokine TGF- β 1 would enhance the suppressive effects of Treg. Elevated levels of TGF- β can be found in BALF of asthma patients, suggesting a pathogenetic role for TGF- β in asthma (8, 9). In addition, overexpression of TGF- β 1 in CD4⁺ T cells induced by retroviral transduction has previously resulted in a marked decrease of airway inflammation and AHR (12), as has direct tracheal administration of TGF- β (20). Impairment of TGF- β signaling in T cells, in contrast, has led to increased allergic airway responses in transgenic mouse models (10, 11). To gain further insight into the specific effects of TGF- β on AHR and airway inflammation, we used the transgenic hCD2-TGF- β 1 mouse model with T cells overexpressing a constitutively active form of TGF- β 1 (26).

Transgenic hCD2-TGF- β 1 mice as well as WT recipient mice receiving 3.5×10^5 transgenic Treg were completely protected from the induction of Ag-dependent AHR. Using transfer of transgenic Treg as well as CD25⁺ T cells overexpressing TGF- β 1, we could demonstrate that the context and not the degree of TGF- β 1 production is essential for its effects on AHR: only mice transferred with transgenic Treg were protected from AHR and had high levels of TGF- β 1 in BALF, whereas transfer of transgenic CD4⁺CD25⁺ T cells resulted in high levels of TGF- β 1 in BALF, but not in protection from AHR. Membrane-bound TGF- β has been implicated in the suppressive function of Treg and may be differentially expressed in transgenic Treg and CD25⁺ T cells, but this has not been further assessed in this study (34).

To gain further insight into the mechanism of protection, hCD2-TGF- β 1 mice were crossed to hCD2-dkT β R11 mice, giving animals with T cells overexpressing active TGF- β 1, but unresponsive

to the effects of TGF- β 1 due to overexpression of a dominant-negative TGF- β type II receptor. WT mice receiving dTG Treg were completely protected from AHR, indicating that TGF- β 1 does not act on transferred Treg themselves in an autocrine manner. These findings are in accordance with reports that impaired TGF- β signaling in T cells does not necessarily abrogate their ability to act as functional Treg (35). To further investigate the mechanisms involved in AHR protection, TGF- β 1 overexpressing Treg were transferred into mice with impaired TGF- β signaling in T cells. These mice were still protected from AHR, either suggesting that cells other than T cells are the target of TGF- β or that other cytokines are the main effectors of AHR protection in this setting.

Suppression of AHR has been described after endotracheal transfer of naturally occurring lung Treg which were shown to produce high levels of TGF- β and IL-10 (36). Therefore, IL-10 may be another key cytokine involved in airway regulation. We have previously demonstrated that overexpression of TGF- β 1 in transgenic hCD2-TGF- β 1 Treg induced the expression of IL-10 (26). Both cytokines seem to be involved in the regulation of airway responses (11, 37) but the understanding of their specific contribution to the modulation of AHR is limited. We therefore used this model to dissect the specific contributions of TGF- β 1 and IL-10 alone or in combination to the suppressive effects on AHR.

Systemically, IL-10 seems to promote AHR, since IL-10^{-/-} mice showed significant protection from MCh-induced AHR, as previously reported (13, 38). However, we could demonstrate that IL-10 produced by transgenic Treg was the main contributing factor leading to complete protection from the induction of AHR in IL-10^{-/-} mice. To further investigate the contribution of IL-10 to the protection from AHR in WT mice, hCD2-TGF- β 1-transgenic mice deficient in IL-10 were adoptively transferred into WT mice. These Treg, overexpressing active TGF- β 1 but deficient in IL-10, did not provide protection from AHR. As has been reported in other *in vivo* models (6, 36), these results indicate that the expression of TGF- β 1 and IL-10 is necessary for the *in vivo* suppression of AHR by Treg. However, basal expression of IL-10 by Treg may be sufficient to maintain their suppressive capacity, since hCD2-TGF- β 1 $\times$ hCD2- Δ kT β R11 dTG Treg were able to confer protection from AHR, although their IL-10 production has been shown to be similar to WT cells (26). Therefore, it seems that IL-10 and TGF- β have synergistic effects in the protection from AHR by Treg and it will be interesting to see in future experiments whether IL-10 without TGF- β is able to mediate protection from AHR by transferred Treg. Clearly, further research is needed into the mechanisms of AHR protection by IL-10, especially to investigate whether IL-10 directly acts on effector T cells or via APCs (39, 40) and whether there are direct effects of TGF- β 1 and IL-10 on airway smooth muscle cells (ASM). Both cytokines have been shown to inhibit chemokine expression in ASM (41–43) and, in humans, adhesion of T lymphocytes to ASM has been found to increase AHR (44).

TGF- β 1 is not only known for its anti-inflammatory, but also for its proinflammatory effects in the early phase of inflammation, as demonstrated by its chemotactic effects on eosinophils (45–47). It was therefore not surprising to find increased airway inflammation in hCD2-TGF- β 1-transgenic mice. Also, recent data suggest that TGF- β 1 is required for the differentiation of Th17 cells (48). TGF- β -secreting T cells may therefore induce a proinflammatory cytokine milieu and thus promote tissue inflammation (49–51). In addition, TGF- β 1 has been implicated in airway remodeling (52). Therefore, it seems prudent to investigate the mechanism of AHR protection by Treg and the specific effects on the early and late

phase of allergic airway disease before further evaluating TGF- β 1 expressing Treg as a therapeutic tool in asthma.

In conclusion, these data demonstrate that Ag-dependent AHR, a hallmark of asthma, can be efficiently suppressed by Treg in vivo. The suppressive capacity of TGF- β 1 overexpressing Treg seems to depend on the expression of IL-10 by Treg themselves but not on TGF- β signaling in Treg or in effector T cells. In our model, Treg were not able to suppress airway inflammation. The elucidation of the mechanisms leading to AHR suppression by Treg may lead to targeted treatment strategies for the early phase of allergic airway responses but further insight into the effects of Treg on airway inflammation is needed.

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Disclosures

The authors have no financial conflict of interest.

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