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Regulatory T Cells More Effectively Suppress Th1-Induced Airway Inflammation Compared with Th2

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Asthma is a syndrome with different inflammatory phenotypes. Animal models have shown that, after sensitization and allergen challenge, Th2 and Th1 cells contribute to the development of allergic airway disease. We have previously demonstrated that naturally occurring regulatory T cells (nTregs) can only marginally suppress Th2-induced airway inflammation and airway hyper-responsiveness. In this study, we investigated nTreg-mediated suppression of Th2-induced and Th1-induced acute allergic airway disease. We demonstrate *in vivo* that nTregs exert their suppressive potency via cAMP transfer on Th2- and Th1-induced airway disease. A comparison of both phenotypes revealed that, despite similar cAMP transfers, Th1-driven airway hyperresponsiveness and inflammation are more susceptible to nTreg-dependent suppression, suggesting that potential nTreg-based therapeutic strategies might be more effective in patients with predominantly neutrophilic airway inflammation based on deregulated Th1 response. *The Journal of Immunology*, 2011, 186: 000–000.

Over the past 20 years, the prevalence and morbidity of allergic diseases have increased dramatically. Allergic asthma is characterized by chronic inflammation of the airways, airway hyperresponsiveness (AHR), mucus production, airway obstruction, and airway remodeling in chronic disease. Th2 cells are thought to predominantly orchestrate the allergic inflammation in the airways and especially to induce eosinophilic airway disease (1–3). However, in recent years it has become clear that the term asthma comprises a rather heterogeneous group of different phenotypes that are induced by divergent pathophysiologic pathways (4). Clinically different inflammatory phenotypes have been identified, including eosinophilic and neutrophilic asthma. Increased levels of IFN- γ have been found in patients with severe asthma, suggesting increased Th1 responses (5, 6). In animal models, it has been demonstrated that Th2 and Th1 cells contribute to the development of an Ag-specific airway inflammation and AHR by the secretion of proinflammatory Th1-cytokines such as IFN- γ and by Ig class switching to IgG2a (7). Thus,

Th1 and Th2 responses need to be evaluated when assessing potential new therapeutic targets.

Studies focused on the functionality of naturally occurring regulatory T cells (nTregs) revealed that these cells have a direct suppressive influence on effector T cells. In principle, nTreg-mediated suppression is characterized by the inhibition of IL-2 gene expression in responder T cells. *In vitro* studies showed that one mechanism of this inhibition is a consequence of the transfer of cAMP from nTregs into responder T cells via gap junctions (8). Cytosolic cAMP in the suppressed T cell can be degraded by phosphodiesterases to 5'-AMP and among all isoforms phosphodiesterase type 4 was found to be the predominant isoenzyme in inflammatory cells (9–12). Recently, we showed that nTregs only moderately suppress Th2-induced allergic airway disease *in vivo*, and treatment with phosphodiesterase type 4 inhibitors substantially enhanced the potency of nTregs in regulating Th2-induced AHR and airway inflammation (13). However, the effect of nTregs on Th1-mediated allergic airway disease was insufficiently studied until now.

In this study we demonstrate, using a strictly T cell-dependent animal model of an acute allergic airway disease, that nTregs exert their suppressive capacity via cAMP transfer on Th2-induced and Th1-induced allergic airway disease. Comparison of the suppressive potency on both phenotypes revealed that Th1-mediated inflammation and AHR are more susceptible to nTreg-dependent suppression than the Th2-driven disease. These findings indicate differences in the impact of the cAMP-mediated suppressive mechanism on Th1 and Th2 cells.

Materials and Methods

Mice

BALB/c Rag2-deficient (Rag2^{-/-}) mice, OVA TCR transgenic BALB/c DO11.10 CD90.1⁺ mice and DO11.10 Rag2^{-/-} CD90.2⁺ mice were bred in the Zentrale Tierzuchtanstalt of the Johannes Gutenberg-University Medical Center, Mainz, Germany. For the experiments, female mice were used at the age of 8–12 wk. Animal procedures were performed according to the current institutional guidelines and conducted in accordance with the Helsinki convention for the use and care of animals.

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Abbreviations used in this article: AHR, airway hyperresponsiveness; BAL, bronchoalveolar lavage; iTreg, induced regulatory T cell; MCh, methacholine; nTreg, naturally occurring regulatory T cell; PAS, periodic acid-Schiff; R_L, airway resistance.

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Cytokines, Abs, and reagents

Mouse rIL-4 was affinity purified using a column with anti-mIL-4 (11B11) mAb bound to sepharose. Hybridoma cells producing anti-CD4 mAb GK1.5 were obtained from American Type Culture Collection (ATCC TIB 207; Manassas, VA). Anti-mIL-4 mAb 11B11 was a gift from Dr. W. Paul (National Institutes of Health, Bethesda, MD) (14). Anti-mIL-4 mAbs BVD4-1D11 and BVD6-24G2 were gifts from Dr. A. O'Garra (National Institute for Medical Research, Mill Hill, U.K.). PE-labeled rat anti-CD25 (7D4) was purchased from BD Biosciences (Heidelberg, Germany). In addition, the following mAbs were used: anti-CD3 mAb 145-2C11 (15), anti-CD28 mAb 37.51 (16), and anti-IFN- γ mAb XMG1.2 (17). If required, mAbs were affinity purified using protein G-sepharose (Pharmacia Biotech, Freiburg, Germany).

Preparation of T cell populations

Conventional CD4⁺CD25⁻ T cells and CD4⁺CD25⁺ T cells (nTregs) were isolated from splenocytes of TCR transgenic DO11.10 mice by positive selection using MACS (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. For purity of the populations MACS sorting of each CD4⁺CD25⁻ and CD4⁺CD25⁺ T cells were performed twice. Conventional CD4⁺CD25⁻ T cells were subsequently depleted from CD4⁺CD25⁺ T cells using mAb PC61 and enriched to a purity of >99%. CD4⁺CD25⁺ enriched T cells were additionally depleted from CD8⁺ T cells, B cells, and macrophages using adequate Dynabeads (anti-CD8, anti-B220, anti-Mac1; Invitrogen/Dynal, Karlsruhe, Germany) and the purity of resulting CD4⁺CD25⁺ T cells was typically >95% (18).

Differentiation of Th cell subsets

Conventional CD4⁺CD25⁻ T cells (1×10^6 cells/ml) were stimulated for 3 d with plate-bound anti-CD3 mAb (3 μ g/ml) and anti-CD28 mAb (10 μ g/ml) in IMDM (Life Technologies) supplemented with 2 mM L-glutamine, 1 mM pyruvate, 5×10^{-5} M β -mercaptoethanol, 10 IU penicillin, 100 μ g/ml streptomycin, and 5% FCS inactivated at 56°C. Appropriate cytokine cocktails were used to polarize these cells into Th1 or Th2 subsets. Th1 cells were differentiated by supplementing the medium with 1 ng/ml mIL-12, 100 U/ml Proleukin (hIL-2) and 1 μ g/ml anti-IL-4 mAb. On day 3, cells were harvested and cultured in the presence of 100 U/ml Proleukin. Th2 cells were polarized by adding 1×10^4 U/ml rIL-4, 10 ng/ml rIL-6 and 20 μ g/ml anti-IFN- γ (XMG1.2) mAb. After 3 d, cells were harvested and cultured in medium containing 200 U/ml rIL-4, 100 U/ml Proleukin, and 100 U/ml rIL-6.

Expansion of CD4⁺CD25⁺ regulatory T cells

Freshly isolated CD4⁺CD25⁺ T cells (nTregs) were cultured in IMDM (Life Technologies) supplemented with 100 ng/ml rIL-2, 2 mM L-glutamine, 1 mM pyruvate, 5×10^{-5} M β -mercaptoethanol, 10 IU penicillin, 100 μ g/ml streptomycin, and 5% FCS inactivated at 56°C. Dynabeads Mouse CD3/CD28 T Cell Expander (Invitrogen/Dynal) were used for expansion during the first 3 d of culture. For an additional 2 d, nTregs were cultured in the supplemented medium described above. Before transfer, frequency of KJ1-26⁺, Helios⁺ and Foxp3⁺ cells was assessed. Helios expression allows the discrimination between CD4⁺CD25⁺Foxp3⁺ nTregs and induced regulatory T cells (iTregs) (19). In the current study, FACS analysis before transfer revealed that, of the expanded CD4⁺CD25⁺Foxp3⁺ cells, >72% expressed KJ1-26 and all CD4⁺CD25⁺Foxp3⁺ cells stained positive for Helios, demonstrating that only nTregs were transferred in this system.

Coculture of T cells

DO11.10 Th1 cells and Th2 cells (2×10^5 cells/ml) were restimulated by anti-CD3 (145-2C11; 3 μ g/ml) and mitomycin C-treated (60 μ g/ml/ 10^7 cells, 30 min) A20 B-tumor cells (2×10^4 /ml) as accessory cells in the presence or absence of in vitro expanded nTregs. The production of IL-4 by Th2 cells and the production of IFN- γ and IL-2 by Th1 cells were assessed by ELISA after 24 h of culture. For proliferation assays, [^{3} H]TdR was added after 96 h of culture, and thymidine uptake was assessed by β scintillation counting after an additional 18 h. Percent inhibition of proliferation was calculated as follows: $100 - [(\text{cpm of coculture "Th1 or Th2 + nTreg"} \div (\text{cpm of Th1 or Th2}) \times 100)]$.

Cytokine assays

IL-4 production by Th2 cells and Th1-secreted IFN- γ and IL-2 were determined by specific two-site ELISA with reference standard curves using known amounts of the cytokine. For the detection of IL-4 we used the following combinations of mAbs: BVD4-1D11 and BVD6-24G2 (20). To

detect IFN- γ in the supernatants of the coculture, the mAbs R4-6A2 and AN18.17.24 were used. IL-2 concentrations in the culture supernatants were assessed using a combination of the mAbs JES6-1A12 and biotinylated JES6-5H4.

Percent inhibition of cytokine production was calculated as follows: $100 - [(\text{cytokine concentration of coculture "Th1 or Th2 + nTreg"})/(\text{cytokine concentration of Th1 or Th2}) \times 100]$.

cAMP ELISA

To determine cytosolic cAMP contents upon coculture with nTregs, CD90.2⁺ Th1 cells and Th2 cells (from DO11.10 Rag2^{-/-}) were sorted 4 h after stimulation in the presence or absence of DO11.10 CD90.1⁺ nTregs according to their CD90.2 expression using a FACS Vantage SE and CellQuest Pro (BD Biosciences), with exclusion of dead cells by propidium iodide incorporation. After sorting, Th cells were washed three times in ice-cold PBS, lysed in 0.1 N HCl (10^7 cells/ml) and a cAMP-specific ELISA (Correlate EIA Direct cAMP Enzyme Immunoassay Kit; Assay Design) was performed according to the manufacturer's instructions. The purity of the sorted cells was always >99%.

Experimental protocol

On day 0, either PBS or $5 \times 10^5 - 2 \times 10^6$ nTregs were injected i.v. into Rag2^{-/-} mice. On day 7, mice received 2×10^6 Th1 cells and 2×10^6 Th2 cells i.v., respectively. Mice were then challenged via the airways by OVA nebulization (1% in PBS) for 20 min daily on days 8 to 13 using an ultrasonic nebulizer (NE-U17; Omron, Hoofddorp, The Netherlands). On day 14, airway reactivity was assessed and animals were killed on the same day for the collection of blood, bronchoalveolar lavage fluid, and lung tissue. In some experiments, mice received 2×10^6 CD90.1⁺ nTregs on day 0 and 2×10^6 CD90.2⁺ Th1 or Th2 cells on day 7 labeled with CFSE (CFDA, Invitrogen, Eugen). Following OVA challenge on days 8 to 11, tracheal lymph nodes were dissected on day 11, and proliferation of CD90.2⁺ cells was assessed by FACS analysis. Percent inhibition of proliferation was calculated as follows: $100 - [(\text{percentage of cells with } >4 \text{ divisions})/(\text{percentage of cells with } <4 \text{ divisions}) \times 100]$.

Measurement of airway reactivity

Measurement of airway resistance (R_L) was conducted on anesthetized, intubated, and mechanically ventilated mice (FlexiVent, Scireq) in response to increasing doses of inhaled bronchoconstrictor methacholine (MCh; 6.25, 12.5, 25, 50, and 100 mg/ml). Measurements of R_L were performed every 15 s after each nebulization step until a plateau phase was reached.

Ex vivo MACS-based cell sort

Mice were treated according to the experimental protocol described above. On day 11, 2 h after the fourth allergen challenge, isolated lungs were minced and exposed to enzymatic digestion by 0.5 mg/ml collagenase type IA (C9891; Sigma-Aldrich) in PBS in a 37°C water bath. After 1 h of incubation, a single-cell suspension was achieved by pushing the digested lung tissue fragments through a 0.9×40 -mm canula (BD Microlance) and a 70- μ m nylon cell strainer (BD Falcon). RBCs were removed using EDTA containing Gey's lysis buffer. CD90.2⁺ Th1 and Th2 cells respectively were purified by MACS-based cell sorting using CD90.2 Microbeads (Miltenyi Biotec) according to the manufacturer's instructions. The purity of sorted Th cells was >98%.

Bronchoalveolar lavage fluid

After measurement of airway reactivity, lungs were lavaged by pressing 1×1 ml PBS via the tracheal tube. Numbers of bronchoalveolar lavage (BAL) cells were assessed using trypan blue dye exclusion. Differential cell counts were performed from cytocentrifuged BAL preparations fixed and stained with the Microscopy Hemacolor Set (Merck). Relative and absolute cell numbers of each cell type were calculated.

FACS analysis

Prior to cell staining unspecific binding of Ab was excluded by using Fc-Block (anti-CD16/32 mAb; BD Pharmingen). Frequencies of CD4⁺CD25⁻Foxp3⁻ and CD4⁺CD25⁺Foxp3⁺ cells in lung tissue and BAL fluid were determined by flow cytometric analysis using PE-Cy7-conjugated rat anti-mouse CD4 (L3T4), PE-conjugated rat anti-mouse CD25 (PC61; both purchased from BD Pharmingen) and allophycocyanin-conjugated hamster anti-mouse/rat Foxp3-staining set (FJK-16s; eBioscience). To discriminate iTregs from nTregs, Helios expression of the expanded nTregs was determined by PE-conjugated hamster anti-mouse/human Helios (22F6;

BioLegend) and PerCP-Cy5.5 conjugated anti-CD25 (PC61; BD Pharmingen) Abs. Frequencies of T cells bearing the OVA transgenic TCR were measured using FITC-conjugated anti-mouse DO11.10 TCR Ab (KJ1-26, eBioscience). Prior to intracellular cytokine staining, cells were stimulated in supplemented cytokine free medium with 50 ng/ml PMA, 750 ng/ml ionomycin, and 1 μ g/ml brefeldin A (all purchased from Sigma-Aldrich) at 37°C. After 4 to 6 h, cells were harvested, washed, and surface stained. Fixation and permeabilization were performed using the BD Cytofix/Cytoperm Fixation/Permeabilization Solution Kit (BD Biosciences). Allophycocyanin-conjugated rat anti-mouse IFN- γ (XMG1.2) and PE-conjugated rat anti-mouse IL-4 (11B11) Abs (all from BD Pharmingen) were used for intracellular cytokine staining.

Histology

For histologic analyses, lungs were fixed by inflation and immersion in 4% formalin and embedded in paraffin to cut tissue sections. The tissue was stained with H&E and periodic acid-Schiff (PAS). Examination of the slides was performed in blinded fashion by two experienced observers using a microscope (BX40, Olympus). On H&E-stained slides, peribronchial and perivascular inflammation of five randomly chosen areas for each slide was graded by a semiquantitative score (no inflammation = 0–4). PAS-stained slides were used to determine the number of mucus-containing goblet cells per millimeter of basement membrane using the imaging software (analysis, Soft Imaging Systems, Stuttgart, Germany).

Statistical analysis

ANOVA was used to determine the levels of difference between all groups. Differences in responsiveness to MCh were assessed by repeated measures ANOVA. Comparisons for all pairs were performed by the Tukey–Kramer honest significant difference test. The number of eosinophils, histology score, and the number of PAS-positive cells were initially analyzed by nonparametric ANOVA (Kruskal–Wallis Test) for overall differences. In case of significant results, the Mann–Whitney *U* test was used to elucidate which specific differences were statistically significant. The *p* values for significance were set at 0.05. Values for all measurements are expressed as the mean $\pm$ SEM.

Results

*n*Tregs more effectively suppress Th1-induced AHR compared with Th2-induced airway reactivity

Th1, Th2, and *n*Tregs were isolated from BALB/c DO11.10 mice, cultured as described in *Materials and Methods*, and transferred to B cell- and T cell-deficient Rag2^{−/−} mice. Airway function was measured 24 h after allergen exposure for 6 d. Baseline values of R_L were not statistically different between the groups studied. Mice that solely received Th1 or Th2 cells developed increased airway reactivity to MCh compared with PBS control mice, which

did not receive any cells. Transfer of *n*Tregs prior to Th2 transfer and allergen challenge lead to a significant but small reduction of AHR (Fig. 1A). In contrast, Th1 recipients that previously received *n*Tregs showed an extensive reduced response in R_L to MCh throughout the dose-response curve compared with mice transferred with Th1 cells only (Fig. 1B).

*n*Tregs effectively suppress Th1-mediated neutrophil infiltration but not Th2-induced eosinophilia

To assess additional characteristic symptoms of allergic airway disease, neutrophils and eosinophils in BAL fluid were quantified. Th1 and Th2 recipients exhibited an increase in total cell counts (data not shown). Transfer of Th1 cells in Rag2^{−/−} mice and allergen challenge of the airways resulted in an accumulation of neutrophils (Fig. 2A), whereas increased numbers of eosinophils were detectable in the BAL fluid of Th2 recipients (Fig. 2B). Previous transfer of *n*Tregs to Th2 recipients had no significant (*p* > 0.05) effects on eosinophil numbers. In contrast, neutrophil numbers were significantly (*p* < 0.01) reduced in Th1 recipients when *n*Tregs were previously transferred.

To further assess lung inflammation, peribronchial inflammation displayed by H&E-stained sections and mucus-producing goblet cells (identified by PAS-positive staining) were analyzed in lung tissue. Th1 and Th2 cell transfer resulted in increased peribronchial inflammation (Fig. 3A, 3B). Application of *n*Tregs significantly (*p* < 0.05) reduced peribronchial inflammation in Th2- and Th1-induced airway inflammation compared with animals that did not receive any *n*Tregs. The reduction was stronger in the Th1 treated animals. The number of goblet cells was significantly higher in animals infused with Th2 cells compared with Th1-treated and PBS-treated animals, in which no PAS-positive cells were detectable. Administration of *n*Tregs resulted in a significant reduction in the number of PAS-positive cells in Th2-transferred animals, but had no effect in mice that received Th1 cells (Fig. 3A, 3C).

To further assess the effectiveness of *n*Treg-mediated suppression on Th1 response, the numbers of transferred *n*Tregs were titrated (2×10^6 , 1×10^6 , and 0.5×10^6). Analyses of airway reactivity, neutrophil infiltration in BAL fluid, and inflammation of the lung tissue via H&E staining concurrently demonstrate that *n*Treg-mediated suppression of Th1-induced AHR and inflammation is a dose-dependent effect and that *n*Tregs exert their influence on airway disease even if induced by 4-fold numbers of Th1 effector cells (Supplemental Fig. 1).

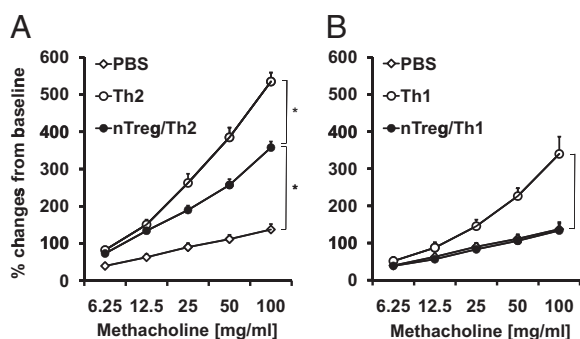


FIGURE 1. *n*Tregs show higher suppressive potency on Th1-induced AHR (B) compared with Th2-mediated AHR (A). R_L was measured in mice 24 h after the last challenge. Administration of Th1 cells (Th1; *n* = 9) and Th2 cells (Th2; *n* = 23) resulted in the development of an increased reactivity to inhaled MCh compared with mice that received only PBS (*n* = 13). Transfer of *n*Tregs before Th2 transfer (*n*Treg/Th2; *n* = 16) reduced AHR in a slight manner. However, the AHR of mice that received *n*Tregs and Th1 cells (*n*Treg/Th1; *n* = 8) was significantly reduced throughout the MCh dose-response curve. **p* < 0.05.

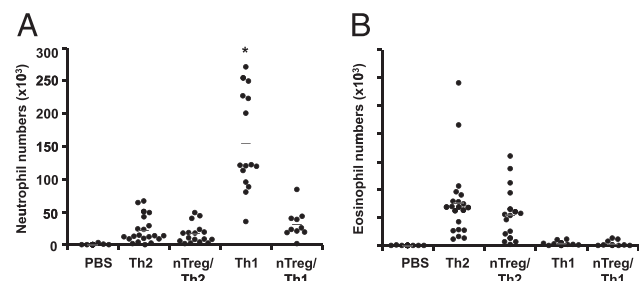


FIGURE 2. Prior *n*Treg transfer has no effect on Th2-induced eosinophil accumulation in BAL fluid, but clearly suppresses Th1-mediated neutrophilic infiltration. Neutrophil (A) and eosinophil (B) numbers in BAL fluid were assessed 24 h after the last airway challenge in mice that received PBS, Th2 cells, *n*Tregs and Th2 cells (*n*Treg/Th2), Th1 cells, and *n*Tregs and Th1 cells (*n*Treg/Th1). Each dot represents a single mouse, and the bar represents the mean. **p* < 0.05 compared with all other groups.

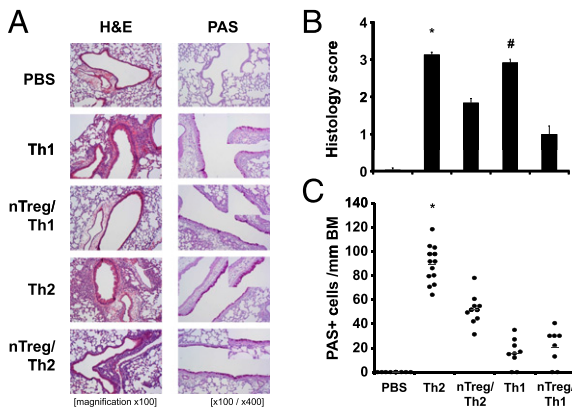


FIGURE 3. Transfer of nTregs efficiently reduces Th1-induced tissue inflammation and goblet cell metaplasia in Th2 recipients. Twenty-four hours after the last airway challenge, tissue inflammation was analyzed using H&E staining to show cell accumulation in parenchymal, peribronchial, and perivascular areas of the lung tissue and PAS staining for goblet cells (A). Adoptive transfer of Th1 and Th2 cells leads to an infiltration of inflammatory cells in the lung tissue compartments compared with the lungs of control mice (PBS), in which no cell infiltration was detectable. Previous administration of nTregs to the Th1-transferred mice (nTreg/Th1) considerably reduces inflammation. In comparison, in the lungs of mice that received nTregs and Th2 cells (nTreg/Th2) inflammation was still present. Original magnification $\times 100$ and $\times 400$ (insets). B, Slides were scored for peribronchial and perivascular inflammation using semiquantitative score from 0 to 4. C, In vivo transfer of nTregs to Th2 recipients reduces goblet cell metaplasia. Numbers of PAS-positive cells were significantly higher in mice transferred with Th2 cells compared with Th1 recipients and the PBS group where no goblet cells were detectable. Additional nTreg transfer significantly reduced numbers of mucus-producing cells, but had no effect in Th1 recipients. The numbers of mucus-producing cells scored 1 per millimeter of basement membrane were quantified for each group as described in *Materials and Methods*. Mean $\pm$ SEM are given. * $p < 0.05$, compared with nTreg/Th2, # $p < 0.05$, compared with nTreg/Th1.

nTregs effectively suppress the proliferation and accumulation of Th1 and Th2 cells in the lung

Based on the effects on airway inflammation and AHR, we assessed the effect of nTregs on OVA-specific CD4⁺ T cell recruitment to the lung and T cell proliferation in vivo. To this end, we assessed frequencies of IL-4- or IFN- γ -producing CD4⁺ T cells by intracellular cytokine staining in Th1, nTreg/Th1, Th2, and nTreg/Th2 transferred mice (all groups, $n = 4$). Indeed, upon gating on CD90.2⁺ T cells, lower frequencies of CD4⁺IFN γ ⁺ or KJ1-26⁺ IFN γ ⁺ cells were detected in nTreg/Th1-transferred mice (mean $\pm$ SEM $1.25 \pm 0.28\%$ or $1.01 \pm 0.22\%$, respectively) compared with Th1-transferred mice ($2.94 \pm 0.63\%$ or $2.65 \pm 0.6\%$). Similarly, numbers of CD4⁺IL-4⁺ or KJ1-26⁺IL-4⁺ were reduced in nTreg/Th2-transferred animals ($0.98 \pm 0.11\%$ or $0.87 \pm 0.11\%$) compared with Th2-transferred mice ($3.61 \pm 0.65\%$ or $3.25 \pm 0.6\%$). In addition, proliferation of T cells in regional lymph nodes of Th1- ($n = 4$) and Th2-transferred animals ($n = 4$) was reduced by nTregs. When assessing the percentage of cells with more than four divisions, we found that nTreg administration inhibited proliferation by $68 \pm 4\%$ (mean $\pm$ SEM) in Th1 animals compared with $52 \pm 6\%$ in Th2 animals (Supplemental Fig. 2A, 2B).

More effective nTreg-mediated suppression of Th1-induced airway disease cannot be attributed to higher local presence of nTregs in the lungs or increased cAMP levels in suppressed Th1 cells

To assess whether the increased suppressive effect of nTregs on Th1-mediated airway disease was due to increased numbers of

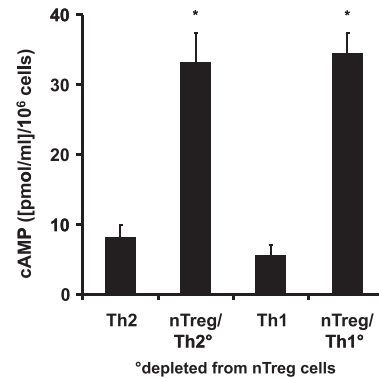


FIGURE 4. In the lung, nTregs transfer similar amounts of cAMP to Th1 and Th2 cells. Two hours after the fourth airway challenge, the lungs of sacrificed mice were dissected; CD90.2⁺ Th1 and Th2 cells were isolated ex vivo and depleted from CD90.1⁺ nTregs using MACS-based cell sorting according to the expression of CD90.2 and CD90.1. The purity of the sorted CD90.2⁺ Th1 cells and Th2 cells was typically $>98\%$. The cytosolic cAMP concentration ex vivo of the MACS-sorted Th cells was determined using a cAMP-specific ELISA. $n = 7$ mice per group were analyzed. * $p < 0.05$, compared with Th2 and Th1.

nTregs in the lung or in the regional lymph node, we analyzed the frequency of CD4⁺CD25⁺Foxp3⁺ lymphocytes. However, the frequencies of CD4⁺CD25⁺Foxp3⁺ cells of total CD4⁺ cells were not statistically different in nTreg/Th1 as compared with nTreg/Th2 transferred animals (Supplemental Fig. 2C, 2D). In previous studies, we have shown that nTreg-mediated suppression of effector T cell responses was dependent on cAMP transfer from nTregs to T effector cells (13). Therefore, the differential potency of nTregs to suppress Th1 or Th2-induced airway disease might be based on a distinct uptake of nTreg-generated cAMP by Th1 and Th2 cells. Consequently, CD90.2⁺ Th1 or Th2 cells were cotransferred with CD90.1⁺ nTregs as described previously (13). After allergen exposure for 4 d, lungs were dissected and CD90.2⁺ Th1 and Th2 cells, depleted from CD90.1⁺ nTregs, and purified ex vivo to a purity $>98\%$. The assessment of intracellular cAMP in the Th cell populations revealed that the concentration of this second messenger was significantly increased when nTregs were present in vivo (Fig. 4). However, no significant differences in cAMP levels of Th1 cells compared with Th2 cells could be observed, excluding the possibility that a differential uptake of

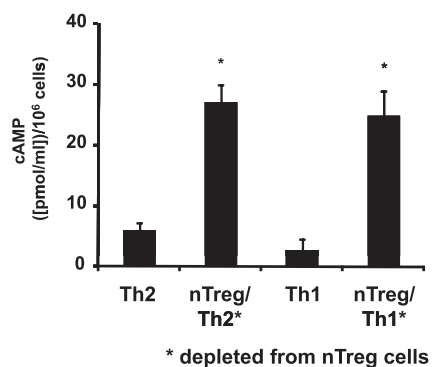


FIGURE 5. The higher susceptibility of Th1 cells to nTreg-mediated suppression is not due to an enhanced transfer of cAMP to the suppressed cell. CD90.2⁺ Th1 cells (1×10^6 cells/ml) and Th2 cells were restimulated in the presence or absence of preactivated CD90.1⁺ nTregs (1×10^6 cells/ml). Four hours after coculture, CD90.2⁺ Th cells were depleted from CD90.1⁺ nTregs by MACS sorting, and cytosolic cAMP content was measured as indicated in *Materials and Methods*. Mean $\pm$ SEM are given from three independent experiments. * $p < 0.05$, compared with Th2 and Th1.

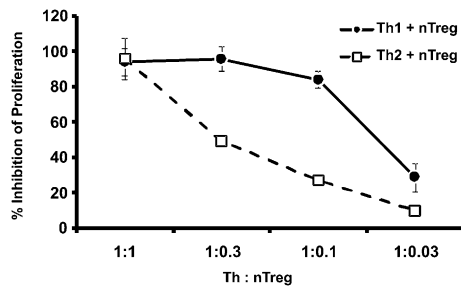


FIGURE 6. The proliferation of Th1 cells is more affected by nTreg-mediated suppression compared with Th2 cell proliferation. Titrated numbers of in vitro expanded nTregs were stimulated with 2×10^5 cells/ml Th1 cells or Th2 cells. [^3H]TdR was added after 96 h of culture, and thymidine uptake was assessed by β scintillation counting after an additional 18 h. Percent inhibition was calculated as described in *Materials and Methods*. Mean $\pm$ SEM from three independent experiments performed in triplicate are shown.

cAMP by Th1 and Th2 cells was responsible for their distinct sensitivity to nTreg-mediated suppression.

Compared with Th2 cells, Th1 cells demonstrate a higher susceptibility to nTreg-mediated suppression in vitro

The data from the in vivo studies suggested that nTregs in the lung can transfer comparable amounts of cAMP to Th1 and Th2 cells. To confirm this assumption, nTregs were cocultured in vitro with Th1 or Th2 cells, and intracellular cAMP was assessed after 18 h. In analogy to the in vivo data, cAMP was transferred in comparable amounts to Th1 and Th2 cells (Fig. 5). However, the proliferation of Th1 and Th2 cells was differentially affected in the presence of nTregs (Fig. 6). The inhibition of Th2-proliferation ceased linearly from 95% at a 1:1 ratio (Th2:nTreg) to 10% at a 1:0.03 ratio. In contrast, the inhibition of the Th1-proliferation by nTregs remained stable on a level $>80\%$, and proliferation did not decline until a ratio of 1:0.03 (Th1:nTreg) was reached. In general, the same finding held true for nTreg-mediated inhibition of Th1- and Th2-derived cytokine production (Fig. 7). Whereas the production of IL-4 was not inhibited at a ratio of 1:0.03 (Th2:nTreg), the production of IFN- γ was still significantly reduced at the same ratio in suppressed Th1 cells. Likewise, the IL-2 production of Th1 cells was also strongly suppressed by nTregs (Fig. 7). Therefore, cytokine production and proliferation of Th1 cells are more strongly affected by nTregs than cytokine production and

proliferation of Th2 cells. This differential sensitivity of Th1 and Th2 cells to nTreg-mediated suppression obviously leads to a distinct efficiency of nTregs observed in regard to the inhibition of asthmatic symptoms induced by Th1 or Th2 cells.

Discussion

Th2-mediated inflammatory responses are often used to model allergic airway disease based on characteristic features of eosinophilic airway inflammation and AHR. Indeed, development of asthma was accounted to an Th1–Th2-imbalance toward predominant Th2 responses (1, 21–25). Because Th1 cells inhibit the proliferation of Th2 cells and IFN- γ inhibits IgE synthesis (26, 27), it has been suggested that promotion of Th1 responses in the lung may prevent asthma (28, 29). However, in recent years progress has been made in identifying different phenotypes of asthma, revealing that, in addition to eosinophil-dominated inflammation, neutrophilic inflammation may also occur in patients with asthma (4), especially in patients with severe asthma (30). Studies in animal models showed that Th1 and Th2 cells are recruited to the lung of sensitized and challenged mice, and it has been demonstrated that allergen-specific Th1 cells are able to cause severe inflammatory responses in the airways after allergen exposure (7, 31). Recent studies have further shown that allergen challenge of sensitized mice leads to a simultaneous upregulation of Th1 and Th2 genes (32). In addition, increased expression of Th1 cytokines has been reported in sputum in patients with exceptionally severe asthma (5, 6), and increased activation of IFN- γ -responsive STAT1 transcription factor has been found in the bronchial epithelial cells of these patients (33).

After transfer to immune-deficient recipient mice, allergen-specific Th1 and Th2 cells persist in vivo, and exposure to allergen aerosol results in lung inflammation. Th2 cells induced airway eosinophilia and increased mucus production, whereas Th1 cells induced neutrophilia in the absence of goblet cells (2, 34). Other groups have reported that Th1 cells can trigger AHR, in addition to airway inflammation (35, 36). In the current study we used an experimental setting of allergen-specific Th cell transfer to B and T cell-deficient Rag2 $^{-/-}$ mice and subsequent airway challenges (2). We found increased airway reactivity in mice transferred with Th1 cells measured by an invasive lung function system. However, reactivity to methacholine was lower compared with the effects observed after Th2 cell transfer. It should be noted that this system is not able to model all aspects of a complex syndrome such as

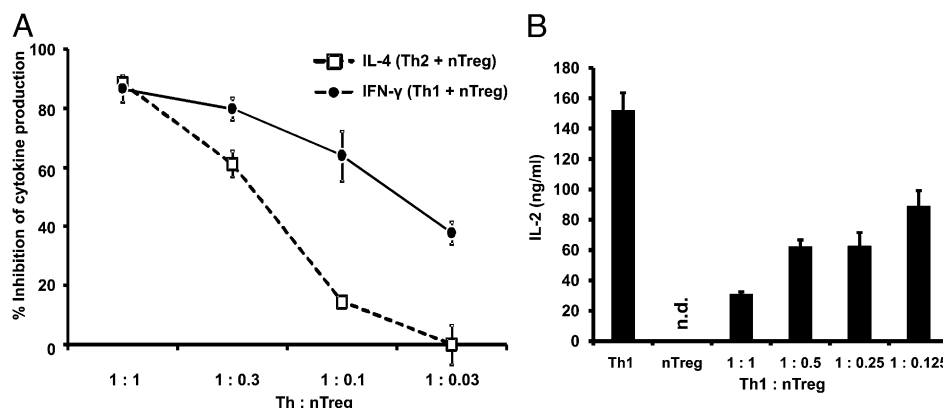


FIGURE 7. A, nTregs have a higher suppressive potency on the cytokine production of Th1 cells compared with Th2 cells. Th1 cells (2×10^5 cells/ml) and Th2 cells were restimulated in the presence or absence of in vitro expanded nTregs in various ratios. After 24 h of culture, the production of IL-4 by Th2 cells and the production of IFN- γ by Th1 cells were assessed by ELISA as stated in *Materials and Methods*. B, IL-2 production of Th1 cells is effectively impaired by nTregs. Th1 cells (2×10^5 cells/ml) were restimulated in the presence or absence of in vitro expanded nTregs in various ratios. After 24 h of culture, the concentrations of IL-2 in the supernatants were assessed by ELISA as stated in *Materials and Methods*.

asthma, but it allows analyzing in vivo interactions of different T cells subtypes, which are important for asthma pathogenesis.

To comparatively study nTreg-mediated suppression of Th1 and Th2 cells in vivo, in vitro expanded nTregs were cotransferred to these animals. Similar to previous reports, transfer of nTregs in addition to Th2 cells suppressed AHR to some degree, moderately decreased tissue inflammation and goblet cell metaplasia, and reduced T cell proliferation in vivo and numbers of IL-4-expressing T cells in the lung (13, 37–39). Remarkably, in Th1-transferred animals, nTregs completely abrogated the development of neutrophilic airway inflammation and AHR and more effectively reduced proliferation in vivo in Th1-transferred animals, indicating a different susceptibility in nTreg-mediated suppression between the Th subtypes. This finding is remarkable, especially considering that Th1-driven airway disease is steroid resistant compared with Th2-induced disease (40). Given the lack of effectiveness of corticosteroids in some patients with asthma (13), new treatment approaches including modulation of nTregs should be considered, especially when patients show an increased expression of Th1 cytokines.

Currently, the effects of nTregs on Th1-induced airway disease are not well understood. However, studies in a model of T cell-induced autoimmune gastritis demonstrated that Th2-induced disease was more resistant to nTreg-mediated suppression compared with proliferative and humoral (IFN- γ) responses driven by Th1 cells (41). One could presume that in a Th1-induced disease, higher numbers of nTregs are present at site of inflammation. However, in the lungs and BAL fluid of challenged mice that received Th1 and nTregs, the frequency of CD4⁺CD25⁺Foxp3⁺ lymphocytes did not differ from animals infused with Th2 and nTregs. Therefore, the varying nTreg-efficiency on CD4⁺ effector subpopulations may be attributed to the suppressive mechanism. Indeed, several mechanisms of suppression used by nTregs have been described in the literature (42–47). Considering that multiple cell types serve as targets of nTreg-mediated suppression and distinct signal transduction pathways partake in these processes, it seems likely that nTregs use multiple means to inhibit immune responses. Interestingly, in murine models of allergic airway disease or colitis, it has been demonstrated that nTreg-derived IL-10 and TGF- β do not contribute to the suppressive potency, but are important in secondary induced suppressor cells in a process termed infectious tolerance (38, 42–44). Seemingly these cytokines are used by secondary iTregs and obviously not by thymus-derived nTregs. In contrast, studies show that one mechanism of nTregs in suppressing CD4⁺CD25⁺ effector T cell function is a consequence of a cell contact-dependent transfer of the key molecule cAMP via gap junctions inhibiting IL-2 expression in the suppressed cell (8, 45–47). Indeed, in vivo nTregs only moderately suppress Th2-induced allergic airway disease. In addition, manipulation of the cAMP metabolism by phosphodiesterase inhibition could confirm that nTregs mediate their suppressive potency through cAMP transfer to the Th2 effector cells in the lung (13). Therefore, we assessed the cytosolic cAMP content of suppressed Th1 and Th2 cells that were sorted ex vivo from the lungs of nTreg/Th1 and nTreg/Th2 reconstituted allergen-challenged Rag2^{-/-} mice. In the presence of nTregs, Th2 and Th1 cells displayed significantly increased contents of cAMP compared with nonsuppressed Th1 and Th2 cells. Strikingly, Th1 and Th2 cells contained similar amounts of cytosolic cAMP when suppressed by nTregs. Therefore, similar amounts of cAMP induce a potent suppression of Th1 responses, whereas Th2 responses were only moderately affected. In analogy to our findings in vivo, in vitro analyses revealed that similar amounts of cAMP were transferred to Th1 and Th2 cells upon coculture with

nTregs. In addition, Th2 cell effector functions remained unaffected by low numbers of nTregs in vitro. Interestingly, nTreg-mediated suppression had a stronger effect on Th1-mediated IFN- γ and IL-2 production. This finding was also reflected by the strong nTreg-mediated inhibition of Th1 proliferation. Corresponding to our findings, Cosmi et al. (48) demonstrated a much lower suppressive activity of human nTregs on the proliferation of Th2 cell clones compared with Th1 cells. They attributed this difference to the ability of Th2 cells to produce cytokines, which sustain their own proliferation even in the absence of responsiveness to IL-2. In addition, an early study by Muñoz et al. (49) revealed that Th1 and Th2 cells differ profoundly in their sensitivity to increased cytosolic cAMP levels. According to our results, high cAMP contents failed to efficiently block Th2-mediated IL-4 production, whereas IL-2 secretion and proliferation of Th1 cells were inhibited.

In summary, development of airway inflammation and AHR can be induced by transferring Th2 and Th1 cells with different inflammatory phenotypes. Furthermore, we demonstrated in vivo that nTregs exert their suppressive potency via cAMP transfer on Th2 and Th1-induced airway disease. A comparison of both phenotypes revealed that, despite similar cAMP transfers, Th1-driven AHR and inflammation are more susceptible to nTreg-dependent suppression, suggesting that potential nTreg-based therapeutic strategies might be more effective in patients with predominantly neutrophilic airway inflammation based on deregulated Th1 response.

Disclosures

The authors have no financial conflicts of interest.

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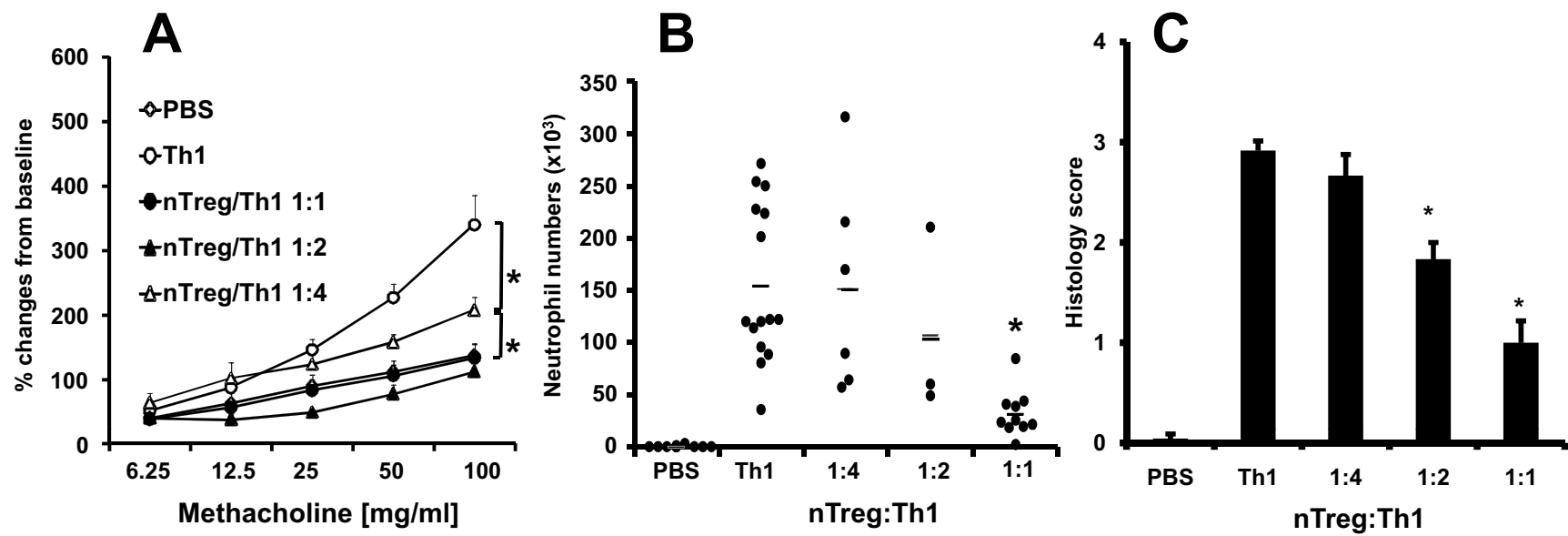
Supplemental figure legends

Figure S1: Effective nTreg-based suppression of Th1-induced AHR and lung inflammation is dose-dependent. Prior to administration of Th1 cells (2×10^6 / mouse) numbers of transferred nTreg cells were titrated (2×10^6 , 1×10^6 and 0.5×10^6 , respectively). Analyses of airway reactivity (A), neutrophil infiltration in BAL fluid (B) and inflammation of the lung tissue via H&E staining (C) demonstrate that nTreg cells exert their influence on airway disease induced by fourfold numbers of Th1 effector cells. Panel A: * $p < 0.05$, panel B and C: * $p < 0.05$ compared to Th1.

Figure S2: Analysis of T cell proliferation *in vivo*. CFSE-labeled CD90.2⁺ Th1 or Th2 cells were injected as described in Material and Methods section. Upon gating on CD90.2-positive cells, proliferation of either Th1 or Th2 cells was assessed by CFSE dilution. Panel A shows a representative example of 2 independent experiments. Panel B shows % inhibition of proliferation of Th1 or Th2 cells in the presence of nTregs (n=4 animals per groups from 2 independent experiments). Means $\pm$ SEM are given. Panel C: Similar frequencies of nTreg cells were found in the lungs after transfer to Th1 and Th2 recipients. Lung cells of mice which either received Th1 cells (Th1), nTreg cells and Th1 cells (nTreg/Th1) or Th2 cells (Th2), nTreg cells and Th2 cells (nTreg/Th2) were isolated 24 h following the last airway challenge. For flow cytometric analyses cells were stained with anti-CD4, anti-CD25 and anti-FoxP3. Frequencies of CD4⁺CD25⁺FoxP3⁺ cells and total cell counts were used to calculate absolute numbers of nTreg cells in the lungs. Each dot represents a single mouse. Mean $\pm$ SEM are shown. * $p < 0.05$ compared to Th2 and Th1. Panel D: Similar frequencies of nTreg cells were found in the tracheal lymph nodes upon transfer to Th1 and Th2 recipients. Lymph nodes of mice which either received Th1 cells (Th1), nTreg cells and Th1 cells

(nTreg/Th1) or Th2 cells (Th2), nTreg cells and Th2 cells (nTreg/Th2) were isolated 24 h following the last airway challenge. To analyze frequencies of nTreg cells among whole lung cells, the cells were stained with anti-CD4, anti-CD25 and anti-FoxP3 and analyzed by Flow cytometry.

Suppl Fig. 1



Suppl Fig. 2

