

Inhibition of cAMP Degradation Improves Regulatory T Cell-Mediated Suppression¹

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Naturally occurring regulatory T cells (nTreg cells) are crucial for the maintenance of peripheral tolerance. We have previously shown that a key mechanism of their suppressive action is based on a contact-dependent transfer of cAMP from nTreg cells to responder T cells. Herein, we further elucidate the important role of cAMP for the suppressive properties of nTreg cells. Prevention of cAMP degradation by application of the phosphodiesterase 4 inhibitor rolipram led to strongly increased suppressive potency of nTreg cells for Th2 cells in vitro and in vivo. Detailed analyses revealed that rolipram caused, in the presence of nTreg cells, a synergistic increase of cAMP in responder Th2 cells. In vivo, the application of nTreg cells in a strictly Th2-dependent preclinical model of asthma had only a marginal effect. However, the additional treatment with rolipram led to a considerable reduction of airway hyperresponsiveness and inflammation in a prophylactic as well as in a therapeutic model. This amelioration was correlated with enhanced cAMP-levels in lung Th2 cells in vivo. Collectively, these data support our observation that cAMP has a key function for nTreg cell-based suppression and they clearly demonstrate that the effect of cAMP on T responder cells can be greatly enhanced upon application of phosphodiesterase 4 inhibitors. *The Journal of Immunology*, 2009, 182: 4017–4024.

Allergic airway disease depends on the induction and effector function of Th2 cells (1). During allergic sensitization, naive CD4⁺ T cells differentiate into allergen-specific Th2 cells, which then, following allergen exposure in the lung, produce effector cytokines like IL-4, IL-5, and IL-13, leading to development of airway inflammation and airway hyperresponsiveness (AHR)⁴ (2). As allergen-specific Th2 cells play a central role in the development and maintenance of airway inflammation, these cells seem to be a promising target for new therapeutic approaches for allergic asthma. Following their “rediscovery” in 1995 (3), regulatory T cells (Treg cells) have been in the focus of recent research. In their functional characterization it has become clear that Treg cells can be subdivided into two subpopulations (4): 1) naturally occurring Treg cells (nTreg cells) that arise from the thymus in dependency of the transcriptional repressor Foxp3 and 2) induced Treg cells (iTreg cells) comprising a rather heterogeneous population of Treg cells, which have in common that they

arise from peripheral naive CD4-positive T cells under variable conditions. The function and suppressive capacity of nTreg cells have been investigated in different models of allergic airway disease. In most of these studies, administration of nTreg cells led to a decrease in miscellaneous features of this disease (5). However, in several studies AHR was either not affected or only partially suppressed by nTreg cell transfer (6–9).

A characteristic feature of nTreg cell-mediated suppression is the inhibition of IL-2 gene expression in responder T cells. This effect is mediated by cAMP transported via gap junction intercellular communication from nTreg cells, containing high intracellular concentrations of cAMP, to responder T cells (10). Hence, cAMP, a general inhibitor of T cell growth, differentiation, and proliferation is central to nTreg cell-mediated immune regulation. Therefore, the modulation of cAMP synthesis and degradation is a promising approach to manipulate nTreg cell-mediated suppression for the treatment of allergic and autoimmune diseases.

Phosphodiesterases (PDE) degrade cAMP to 5'-AMP and PDE4 is the predominant isoenzyme in inflammatory cells. Inhibitors of PDE4 act by increasing intracellular concentrations of cAMP, which has a broad range of anti-inflammatory effects on various effector cells. The effects of PDE4 inhibition on allergic airway disease have been evaluated and several PDE4 inhibitors have been shown to modulate effector cell functions and to reduce inflammation and AHR (11–14). However, the mechanisms underlying the effect of PDE4 inhibitors on allergic airway disease in vivo remain poorly defined.

In this study, we demonstrate that inhibition of Th2 responses by coculture with nTreg cells is considerably enhanced in the presence of the PDE4 inhibitor rolipram. Concomitantly, the cAMP concentration in suppressed Th2 responder cells increased strongly upon treatment with rolipram indicating that cAMP represents a central suppressive mechanism of nTreg cells. Using two strictly Th2-dependent models of allergic airway disease, prophylactic and therapeutic, we further demonstrate that treatment with PDE4

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⁴ Abbreviations used in this paper: AHR, airway hyperresponsiveness; Treg, regulatory T cell; nTreg, naturally occurring Treg cell; iTreg, induced Treg cell; PDE, phosphodiesterase; BAL, bronchoalveolar lavage; R_L, airway resistance; C_{dyn}, dynamic compliance; MCh, methacholine; PAS, periodic acid-Schiff; preTreg, preactivated nTreg; TCC, total cell count.

inhibitors greatly enhance the effectiveness of transferred nTreg cells in controlling airway inflammation and AHR in vivo by directly inhibiting the degradation of cAMP in Th2 cells upon contact with nTreg cells, thus strengthening nTreg cell-mediated suppression.

Materials and Methods

Mice

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

Cytokines, Abs, and reagents

Mouse rIL-4 was affinity purified using a column with anti-mIL-4 (11B11) mAb bound to Sepharose. Human rIL-2 (Proleukin, 18×10^6 IE, Chiron). Hybridoma cells producing anti-CD4 mAb GK1.5 or H129.19 were obtained from the American Type Culture Collection (ATCC TIB 207). Anti-mIL-4 mAb 11B11 was a gift from Dr. W. Paul (National Institutes of Health, Bethesda, MD). 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.). Biotin-labeled rat anti-CD25 (7D4) was purchased from BD Biosciences. PE-conjugated streptavidin was purchased from Dianova. In addition, the following mAbs were used: rat anti-TCR clonotype KJ1-26, anti-CD3 mAb 145-2C11, anti-CD28 mAb 37.51, anti-IFN- γ mAb XMGI.2 (19), PE-Cy7-conjugated anti-CD90.1 (HIS51; BD Pharmingen) and allophycocyanin-conjugated CD90.2 (53-2.1; BD Pharmingen). If required, mAbs were affinity purified using protein G-Sepharose (Pharmacia Biotech) and coupled with FITC or biotin. Mitomycin C was purchased from Sigma-Aldrich (M 0503). CD90.2 MicroBeads (Order no. 130-049-101) were purchased from Miltenyi Biotec. Grade V OVA was purchased from Sigma-Aldrich (A5503). Rolipram was purchased from Sigma-Aldrich (R6520). Acetyl- β -methylcholine chloride (Methacholine) was purchased from Sigma-Aldrich (A2251).

Preparation of T cell populations

Conventional CD4⁺ CD25⁻ T cells and CD4⁺ CD25⁺ T cells (nTreg cells) were isolated from splenocytes of TCR transgenic DO11.10 mice by positive selection using MACS (Miltenyi Biotec) 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 >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) and the purity of resulting CD4⁺CD25⁺ T cells was typically >95% (20).

Generation of Th2 cells

After isolation, 1×10^6 conventional CD4⁺CD25⁻ T cells/ml were differentiated into Th2 cells by stimulation 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, 5×10^{-5} M 2-ME, 1 mM pyruvate, 10 IU penicillin, 100 μ g/ml streptomycin, and 5% FCS inactivated at 56°C, 1×10^4 U/ml rIL-4, IL-6 (1×10^3 U/ml), and 20 μ g/ml anti-IFN- γ mAb (XMGI.2). On day 3, cells were harvested and cultured in the presence of 200 U/ml rIL-4, 100 U/ml proleukin, and 100 U/ml mIL-6 for an additional 2 days (21).

Preactivation of CD4⁺CD25⁺ Treg cells

Isolated CD4⁺ CD25⁺ T cells (nTreg cells) were cultured in IMDM (Life Technologies) supplemented with 2 mM L-glutamine, 5×10^{-5} M 2-ME, 1 mM pyruvate, 10 IU penicillin, 100 μ g/ml streptomycin, 5% FCS inactivated at 56°C, and 1000 U/ml proleukin. Preactivation of these cells occurred during the first 3 days of culture by using Dynabeads Mouse CD3/CD28 T cell Expander (Invitrogen/Dynal). Upon removal of the Dynabeads Mouse CD3/CD28 T cell Expander, preTreg cells were cultured for additional 2 days in the above described supplemented IMDM.

Coculture of T cells

Th2 cells were restimulated in the absence or presence of nTreg cells or preTreg cells using mitomycin C-treated (60 μ g/ml/ 10^7 cells, 30 min) A20

B-tumor cells (10^5 /ml) and 3 μ g/ml anti-CD3 (145-2C11). The production of IL-4 was assessed by ELISA after an additional 24 h. If indicated, cells were stimulated in the presence of 100 nM rolipram (Sigma-Aldrich, R6520).

IL-4 assay

IL-4 was assayed by specific two-site ELISA with reference standard curves using known amounts of this cytokine. For the detection of IL-4 we used the following combinations of Abs: BVD4-1D11 and BVD6-24G2 (22). After measuring IL-4 in the supernatants percent inhibition of IL-4 production was calculated as follows: $100 - [(IL-4 \text{ U/ml Th2 in the absence or presence of nTreg cells/preTreg cells and/or rolipram})/(IL-4 \text{ U/ml Th2}/100)]$.

cAMP ELISA

To assess cytosolic cAMP concentrations upon coculture with nTreg cells, Th2 cells from DO11.10 Rag2^{-/-} CD90.2⁺ were sorted 4 h after stimulation in the presence or absence of nTreg cells (DO11.10 CD90.1⁺) and/or 100 nM rolipram according to their CD90.2 expression using a FACS Vantage (FACSVantage SE and CellQuest Pro; BD Biosciences), with exclusion of dead cells by propidium iodide incorporation. After sorting, Th2 cells were washed three times in ice-cold PBS, lysed in 0.1 N HCl (10^7 /ml), and a cAMP-specific ELISA (Correlate EIA Direct Cyclic AMP Enzyme Immunoassay kit; Assay Design) was performed. The purity of the sorted cells was always >99%.

Experimental protocol

Rag2^{-/-} mice received either PBS or 2×10^6 preTreg cells on day 0 by i.v. injection. On day seven, 2×10^6 Th2 cells were applied by i.v. injection. Mice were then challenged via the airways using nebulized OVA (1% in saline) with an ultrasonic nebulizer (NE-U17; Omron) for 20 min daily on days 8 to 13. Mice were treated 30 min before each OVA challenge with either PBS or rolipram (1 mg/kg body weight) by i.p. injection. On day 14 airway reactivity was assessed and animals were killed on the same day for the collection of bronchoalveolar lavage (BAL) fluid, blood, and lung tissue (see supplemental Figure S1).⁵

Secondary model of allergic airway disease

Ten- to 12-wk-old BALB/c mice were sensitized by i.p. injection of 20 μ g of OVA (Grade V; Sigma-Aldrich) emulsified in 2.25 mg of aluminum hydroxide (AlumImject; Pierce) in a total volume of 100 μ l on days 1 and 14. Mice were challenged (20 min) via the airways with OVA (1% in saline) for 3 days (days 26, 27, and 28; primary challenge) (see supplemental Fig. S2) using ultrasonic nebulization (NE-U17, Omron). Control mice groups received OVA challenge alone. For the secondary challenge protocol, 6 wk after the primary challenge, mice were exposed to a single OVA challenge (1% in PBS; secondary challenge), and airway reactivity and tissues were assessed 48 h later (23, 24).

One day before secondary challenge, mice received either PBS or either 1×10^6 , 2.5×10^6 , or 5×10^6 preTreg cells derived from OVA-transgenic DO11.10 donors. Mice received an i.p. injection of rolipram (0.1 mg/kg body weight) or PBS 2 h before and 6 and 24 h after OVA challenge (14). In supplemental experiments mice received an i.p. injection of piclamilast (0.1 mg/kg body weight) (provided by Christian Hesslinger, Nycomed, Konstanz, Germany) or PBS 2 h before and 6 and 24 h after OVA challenge.

Ex vivo FACS-based cell sort

Mice were treated as described under *Experimental protocol*. On day 11, 2 h after the fourth allergen challenge, animals were sacrificed and lungs were dissected into small pieces and exposed to an enzymatic digestion by 0.5 mg/ml collagenase type IA (Sigma-Aldrich; C9891) in PBS $1 \times$ in a 37°C water bath for 60 min. After enzymatic digestion, a single-cell suspension was produced by pushing the lung tissue fragments through a 0.9×40 mm canula (BD Microlance) and a 70- μ m nylon cell strainer (BD Falcon). Based on the single-cell suspension, CD90.2-positive Th2 cells were first purified from lungs to ~70% by MACS-based cell sorting for CD90.2-positive Th2 cells using CD90.2 Microbeads according to the manufacturers' instructions. Upon staining for CD90.1 and CD90.2, respectively, Th2 cells were further purified by using a FACS-based cell sorter (FACSVantage SE and CellQuest Pro; BD Biosciences), with exclusion of dead cells by propidium iodide incorporation. The purity of the sorted cells was typically >98% (see Fig. 6A).

⁵ The online version of this article contains supplemental material.

Measurement of airway reactivity

Measurements of the airway resistance (R_L) and dynamic compliance (C_{dyn}) were performed on anesthetized, intubated, and mechanically ventilated mice (FlexiVent, Scireq) in response to increasing doses of inhaled methacholine (MCh) (6.25, 12.5, 25, 50, and 100 mg/ml). Measurements of R_L and C_{dyn} were performed every 15 s following each nebulization step until a plateau phase was reached (25).

Bronchoalveolar lavage

After assessment of airway function, lungs were lavaged via the tracheal tube with PBS (1×1 ml). Numbers of BAL cells were counted by using trypan blue dye exclusion. Differential cell counts were made from cyto-centrifuged preparations fixed and stained with the Microscopy Hemacolor Set (Merck). Percentage and absolute numbers of each cell type were calculated. The numbers of $CD4^+CD25^-FoxP3^-$, $CD4^+CD25^+FoxP3^-$, and $CD4^+Foxp3^+$ cells were assessed by flow cytometry analysis (see supplemental Figure S3) using PE-Cy7-conjugated rat anti-mouse CD4 (H129.19), PE-conjugated rat anti-mouse CD25 (PC61) (both purchased from BD Pharmingen) and allophycocyanin-conjugated anti-mouse/rat Foxp3-Set (FJK-16s; eBiosciences). Absolute numbers of CD4-positive, CD25-positive, and Foxp3-positive T cells were calculated by multiplying the percentage with total cell count.

Histology

Lungs were fixed by inflation (1 ml) and immersion in 10% formalin and embedded in paraffin. Tissue sections were stained with H&E and periodic acid-Schiff (PAS). Slides were examined in blinded fashion by two experienced observers with a microscope (BX40, Olympus) and peribronchial and perivascular inflammation was graded by a semiquantitative score (no inflammation = 0 to 4). For each slide, five randomly chosen areas were scored. Using PAS-stained slides, the number of goblet cells was analyzed using the imaging software (analysis, Soft Imaging Systems). The number of mucus-containing cells per millimeter of basement membrane was determined.

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. Number of eosinophils, histology score, and 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

Suppression of Th2 cells by nTreg cells is augmented in the presence of PDE4 inhibitor rolipram

Although nTreg cells are capable in suppressing naive $CD4^+$ T cells, the inhibition of Th2 cells can only be accomplished in vitro by preactivated nTregs (preTreg cells) (15, 16). Regarding the mechanism of suppression, we could recently demonstrate that the transfer of cAMP from nTreg/preTreg cells to suppressed T cells is important for the suppressive potency of nTreg cells, disclosing a key role for cAMP in this process (10). These findings implicated that the prevention of cAMP degradation by PDE inhibitors in suppressed T cells could considerably strengthen the suppressive capacity of nTreg cells.

This assumption could be corroborated by costimulating Th2 cells and nTreg or preTreg cells in the absence or presence of the PDE4 inhibitor rolipram (Fig. 1). Consistent with our previous data, preTreg cells strongly inhibited the production of IL-4 in a concentration-dependent manner, while nTreg cells had a minimal suppressive influence (15, 16). The comparatively low suppressive capacity of nTreg cells increased profoundly in the presence of rolipram and the same held true for preTreg cells when used at lower ratios (1:0.3–1:0.03). Because such low T effector/Treg cell ratios can be usually observed in vivo, application of rolipram seemed to be an attractive option to treat diseases caused by Th2 cells. The treatment of Th2 cells solely with rolipram led to a

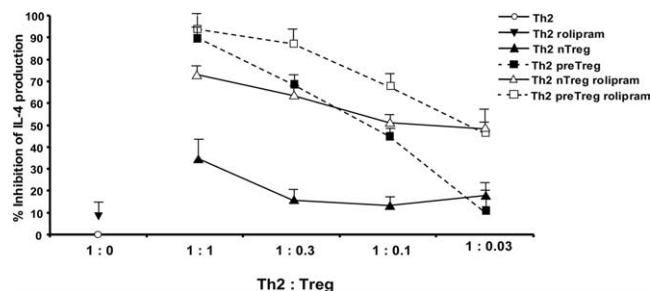


FIGURE 1. PDE4 inhibition in Th2 cells increases the susceptibility to nTreg cell-mediated suppression. Th2 cells (2×10^5 /ml) were activated alone ($\circ$) or together with freshly isolated nTreg cells ($\blacktriangle$) or preactivated nTreg (preTreg) cells ($\blacksquare$) in various ratios in the absence or presence of 300 nM rolipram (Th2 plus rolipram ($\blacktriangledown$); Th2 plus nTreg plus rolipram ($\triangle$); Th2 plus preTreg plus rolipram ($\square$)). IL-4 production was assessed after 48 h and percent inhibition was calculated as stated in the *Materials and Methods* section. Mean $\pm$ SEM from four independent experiments performed in triplicates are shown.

minimal inhibition of IL-4 production (Fig. 1), thus strongly suggesting that nTreg and preTreg cell-mediated suppression can be essentially improved by stabilizing cAMP in suppressed Th2 cells.

To prove this conclusion, the concentration of cAMP was determined in Th2 cells activated in the absence and presence of preTreg cells or rolipram and a combination of both as well. PreTreg cells were depleted from Th2 cells after coculture. They were preferred because they contain strongly elevated concentrations of cAMP as compared with nTreg cells (10). Unlike preTreg cells, rolipram alone only led to a weak increase of cAMP in Th2 cells while rolipram in combination with preTreg cells synergistically increased the concentration of cAMP in Th2 cells (Fig. 2). Treatment solely of preTreg cells with rolipram led to a moderate increase of intracellular cAMP in the range of $\sim 30\%$ (data not shown). Hence, these in vitro studies demonstrated convincingly that the inhibition of PDE4 strongly increases the suppressive potency of preTreg cells most possibly by two mechanisms: 1) as a result of a moderate increase of cAMP in preTreg cells and 2) the retention of a considerable high concentration of transferred cAMP in suppressed Th2 cells by inhibiting the degradation of this second

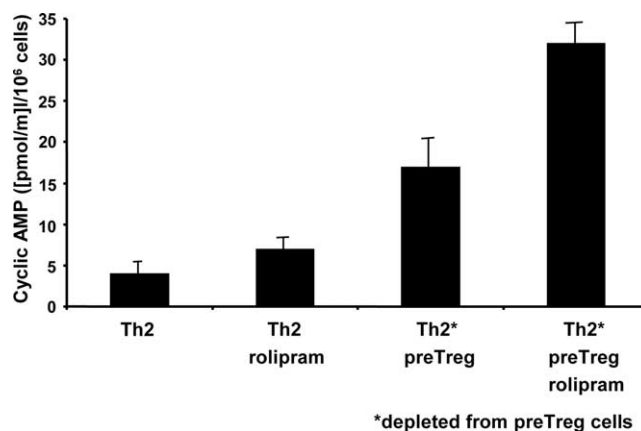


FIGURE 2. PDE4 inhibition augments the suppressive potency of preTreg cells by decreasing cAMP decomposition. $CD90.2^+$ Th2 cells (1×10^6 /ml) were activated in the absence or presence of 300 nM rolipram and in the absence or presence of $CD90.1^+$ preTreg cells (1×10^6 /ml). Four hours upon coculture, $CD90.2^+$ Th2 cells were depleted from $CD90.1^+$ preTreg cells by FACS sorting and cytosolic cAMP content was measured as indicated in *Materials and Methods*. Mean $\pm$ SEM are given of one representative experiment from three experiments.

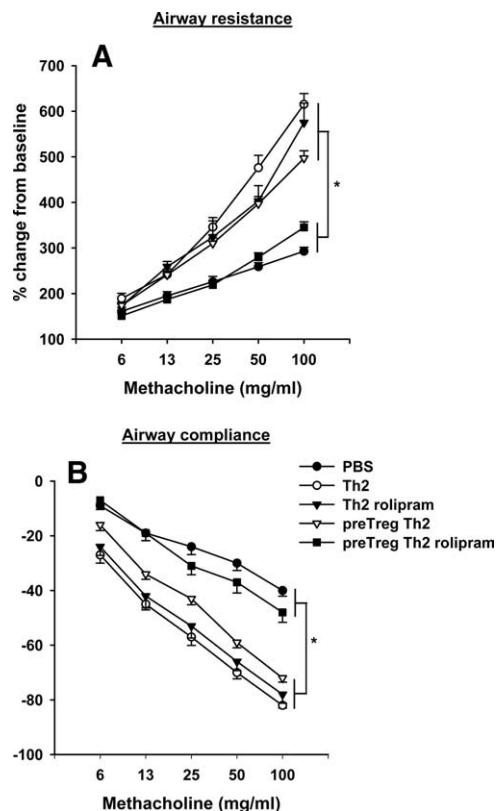


FIGURE 3. PDE4 inhibition augments the suppressive potency of pre-Treg cells on AHR in a prophylactic model of allergic airway disease. Airway responsiveness (R_L panel A and C_{dyn} ; B) were measured in mice 24 h after the last challenge. Mice which received Th2 cells (Th2, $n = 18$) developed increased reactivity to inhaled MCh compared with mice which received only PBS (PBS, $n = 12$). Treatment of Th2-transferred animals with rolipram (Th2 rolipram, $n = 7$) had no effect on airway reactivity. Also transfer of preTreg cells before Th2 cells had no significant effect on airway reactivity (preTreg Th2, $n = 16$). However, transfer of preTreg cells and treatment with rolipram (preTreg Th2 rolipram, $n = 10$) significantly reduced airway reactivity throughout the dose-response curve. Results are from three independent experiments. Mean $\pm$ SEM are given. *, $p < 0.05$.

messenger. As a corollary, the in vivo relevance of these findings was examined using a strictly Th2-dependent model of asthma.

Treatment with PDE4 inhibitor improves the suppressive capacity of preTreg cells on Th2 cell-mediated development of AHR

To assess the effect of PDE4 inhibition on nTreg cell-mediated suppression of Th2 responses in vivo, we used a model of Th2 cell-induced allergic airway disease. As nTreg cells only marginally suppressed Th2 cells in vitro (see Fig. 1) and could hardly be isolated in numbers required for the in vivo experiments, we investigated in the following the effect of in vitro expanded preTreg cells on the development of Th2-induced AHR and airway inflammation. Th2 and preTreg cells were cultured from BALB/c DO11.10 mice as previously described (15) and transferred to T cell and B cell-deficient Rag2^{-/-} recipients. Following allergen exposure for 6 days airway function was assessed. Baseline values of pulmonary resistance (R_L) and dynamic compliance (C_{dyn}) were not statistically different between the groups studied. Mice, which received Th2 cells, developed increased airway reactivity to MCh following airway challenge compared with mice that did not receive any cells before challenge (Fig. 3). Treatment solely with PDE4 inhibitor rolipram following Th2 cell transfer did not sig-

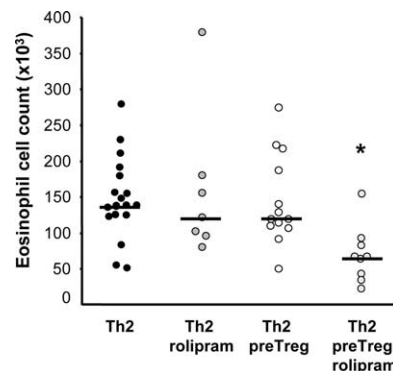


FIGURE 4. Airway inflammation in BAL fluid is significantly reduced upon PDE4 inhibition in the presence of preTreg cells. Eosinophil numbers in BAL fluid were assessed 24 h following the last challenge in mice which received PBS, Th2 cells, Th2 cells and rolipram (Th2 rolipram), Th2 cells and preTreg cells (Th2 preTreg), and Th2 cells and preTreg cells and rolipram (Th2 preTreg rolipram). Each dot represents a single mouse. Bar represents mean. *, $p < 0.05$ compared with all other groups.

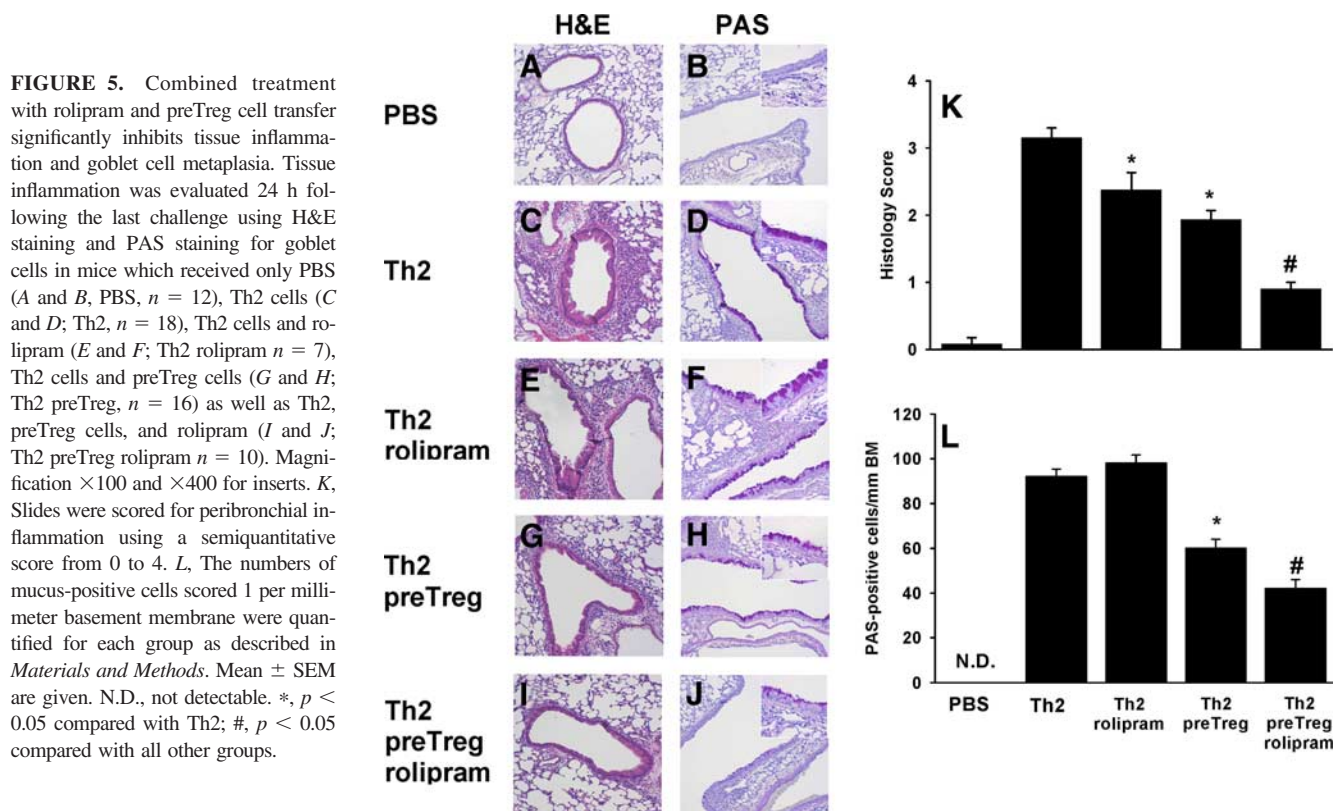
nificantly affect airway reactivity. Also, transfer of preTreg cells before Th2 transfer resulted in a small but not significant reduction of airway reactivity (Fig. 3). In contrast, mice which received pre-Treg cells in combination with rolipram treatment exhibited a considerably ($p < 0.01$) lower response in R_L and C_{dyn} to MCh throughout the dose-response curve compared with mice that received solely Th2 cells. The nearly complete reduction of AHR under the simultaneous influence of rolipram and preTreg cells further corroborated the finding that the suppressive properties of such preTreg cells depend on cAMP that is certainly stabilized in the presence of PDE4 inhibitors.

PDE4 inhibition in addition to preTreg cell transfer reduces Th2 cell induced airway eosinophilia in BAL fluid

In addition to AHR, the accumulation of inflammatory cells in the BAL fluid is a characteristic symptom of allergic airway diseases and was therefore evaluated 24 h after the last airway challenge. Rag2^{-/-} mice that received Th2 cells showed an increase in total cell counts (TCC) (mean $\pm$ SEM, $247 \pm 50 \times 10^3$ cells) and eosinophil numbers (Fig. 4) in BAL fluid compared with mice challenged in the absence of Th2 cells ($53 \pm 20 \times 10^3$ cells), where no eosinophils were detected. Treatment of Th2 cell recipient Rag2^{-/-} mice with rolipram had no effect on TCC ($267 \pm 58 \times 10^3$ cells) and only a minimal effect on eosinophil numbers (Fig. 4). Similarly, transfer of preTreg cells before Th2 cells had little effect on TCC ($223 \pm 38 \times 10^3$ cells) or eosinophil numbers in BAL fluid. However, the combination of preTreg cell transfer and treatment with rolipram resulted in a decrease in TCC ($124 \pm 25 \times 10^3$) and eosinophil numbers compared with mice that received Th2 cells alone or Th2 cells in combination with preTreg cells (Fig. 4).

PDE4 inhibition in addition to preTreg cell transfer reduces Th2 cell-induced goblet cell metaplasia and tissue inflammation

Similarly to the results observed in BAL fluid Th2 transfer and airway challenge resulted in increased peribronchial and perivascular inflammation observed in H&E-stained sections of lung tissue (Fig. 5). Treatment with rolipram alone had little effect on tissue inflammation. Transfer of preTreg cells before Th2 cell administration led only to a moderate decrease in peribronchial and perivascular inflammation. However, transfer of preTreg cells and subsequent treatment with rolipram resulted in a profound decrease of the Th2-mediated inflammatory response (Fig. 5, H & E).



Besides inflammation, also the numbers of goblet cells (PAS positive cells) in the airway epithelium were strongly increased after transfer of Th2 cells and subsequent allergen challenge. Treatment with rolipram did not affect the number of goblet cells. Similar to inflammatory changes, transfer solely of preTreg cells moderately decreased the amount of mucus-producing goblet cells whereas the combination of preTreg cells and rolipram was most effective in reducing the number of these cells in the airways (Fig. 5, PAS).

Treatment with PDE4 inhibitor strongly improves preTreg cell-mediated suppression by preventing cAMP degradation in Th2 cells in vivo

To corroborate our finding that the mechanism of nTreg cell-mediated suppression is based on strongly increased cAMP concentration in target T cells upon cell contact with nTreg cells, we conducted experiments to assess the cAMP content of suppressed T cells directly ex vivo. To this end, CD90.2-positive Th2 cells from BALB/c DO11.10 RAG2^{-/-} mice were transferred to Rag2^{-/-} recipients with or without CD90.1-positive preTreg cells from BALB/c DO11.10 mice as described previously. Following allergen exposure for 4 days in the presence or absence of rolipram lungs were dissected and Th2 cells were purified ex vivo according to their expression of CD90.2 to a purity >98% (Fig. 6A). Assessment of intracellular cAMP by ELISA revealed that in the presence of preTreg cells, this second messenger was significantly increased in Th2 cells. Yet, the coadministration of rolipram and preTreg cells remarkably exceeded this value (Fig. 6B). Thus, these data demonstrate for the first time that preTreg cells strongly raise the content of cAMP in Th2 cells in vivo, which can be further augmented by concomitant inhibition of PDE4.

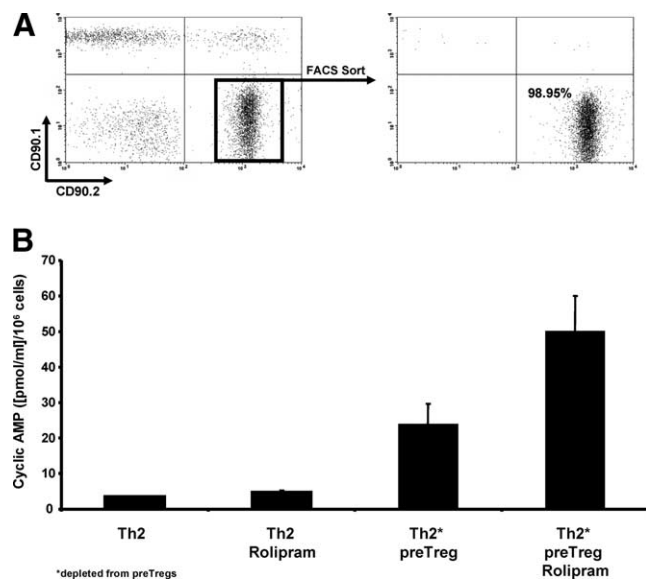


FIGURE 6. In vivo administration of rolipram enhances preTreg cell-mediated suppression by stabilizing cAMP in Th2 cells. After the fourth allergen challenge using the protocol as outlined in *Experimental protocol* in the *Materials and Methods* section, mice were sacrificed, lungs were dissected, and CD90.2⁺ Th2 cells were isolated ex vivo and depleted from CD90.1⁺ preTreg cells using a combination of MACS-based purification and subsequent FACS-based cell sorting according to the expression of CD90.2 and CD90.1. A, To assess the purity after MACS sorting (left dot blot) and the resulting purity after FACS-based cell sort (right dot blot), Th2 cells were analyzed for the presence of contaminating CD90.1⁺ preTreg cells and for their CD90.2 expression, respectively. The purity of the sorted CD90.2⁺ Th2 cells was typically >98%. B, The cytosolic cAMP concentration ex vivo of the FACS-sorted Th2 cells was assessed using a cAMP-specific ELISA. In each experiment $n = 6$ mice per group were analyzed. Mean $\pm$ SEM from two independent experiments are given.

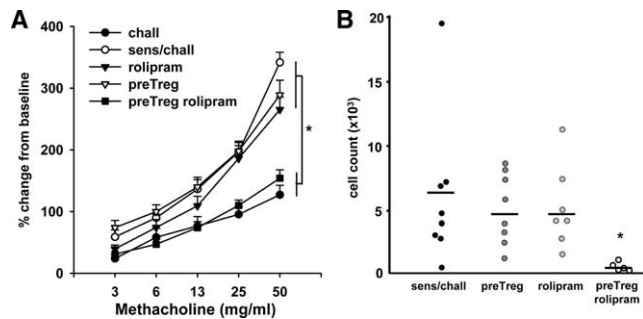


FIGURE 7. PDE4 inhibition augments the suppressive potency of pre-Treg cells in a therapeutic model of allergic airway disease. Airway responsiveness (A) and numbers of eosinophils in BAL fluid (B) were measured in mice 48 h after a secondary airway challenge. Mice which were sensitized and challenged (sens/chall, $n = 9$) showed increased airway reactivity and numbers of eosinophils in BAL fluid compared with only challenged mice (chall, $n = 9$), where no eosinophils were detectable. Transfer of 2.5×10^6 preTreg cells (preTreg, $n = 8$) or treatment with rolipram (rolipram, $n = 7$) of sensitized and challenged mice had no significant effect on airway reactivity and numbers of eosinophils. In contrast, transfer of preTreg cells in combination with rolipram (preTregs rolipram, $n = 5$) effectively suppressed AHR and airway inflammation. A, Means $\pm$ SEM are given. B, Bar represents mean. *, $p < 0.01$.

PDE4 inhibition concomitantly with preTreg cell transfer inhibits allergic airway disease in a therapeutic model of allergic airway disease

To further assess whether transfer of preTreg cells and treatment with PDE4 inhibitors is also effective in a therapeutic setting, we used a previously described secondary model of allergic airway disease (23, 24). In this model, transfer of allergen-specific pre-Treg cells before secondary challenge had only a marginal effect on the development of AHR (see supplemental Figure S4). The same held true for the administration solely of rolipram (Fig. 7A). However, coadministration of preTreg cells with rolipram showed a remarkable decrease in airway reactivity nearly to that observed in unsensitized mice (Fig. 7A). Similarly, treatment with preTreg cells and rolipram considerably reduced number of eosinophils in BAL fluid compared with the sensitized and challenged animals (Fig. 7B), suggesting that the strengthening of nTreg cell-mediated suppression by inhibition of PDE4 is a promising strategy for the treatment of allergic airway diseases. To further confirm that these results were specific for PDE4 inhibition, a different PDE4 inhibitor, piclamilast, was used. Again treatment with preTreg or piclamilast alone had only a minimal effect on AHR and airway inflammation. However, combined treatment with preTreg cells and piclamilast resulted in strong suppression of AHR and eosinophil numbers in BAL fluid (see supplemental Figure S5).

Discussion

Analysis of the suppressive activity of nTreg cells in vitro on allergy- and asthma-promoting Th2 cells revealed that freshly isolated nTreg cells had only a minimal effect while preTreg cells strongly suppressed IL-4 production of Th2 cells (15). The fact that preTreg cells have a strongly increased suppressive potency was originally described by Thornton et al. (17), but the reason for this 4- to 6-fold enhancement of suppressive activity was not clear. Originally our interpretation was that preTreg cells respond with a faster kinetics compared with freshly isolated nTreg cells, thus enabling the suppression of Th2 cells that will also respond with a rapid kinetics (15). An additional possibility is suggested by our finding that preTreg cells contain concentrations of cAMP twice in excess of nTreg cells (10). We and others could show that cAMP

is a key molecule in nTreg cell- as well as preTreg cell-mediated suppression (10, 18, 19, 26). In particular, we were able to show that cAMP is transferred cell contact-dependently via gap junctions to suppressed T cells where it leads to the inhibition of the expression of IL-2. Enhanced concentrations of cAMP in preTreg cells result in higher concentrations of cAMP transferred to cocultured Th2 cells, ultimately leading to a stronger inhibition of such T cells by an increased cAMP level. Furthermore, adenosine produced by nTregs was found to be involved in their suppressive properties (18, 19, 26). As a contribution of adenosine in vivo could not be ruled out, the accumulation of cAMP in Th2 cells could also be promoted by additional pathways.

However, stimulation of T cells through TCR in combination with strong costimulation leads to a recruitment of PDE4 to lipid rafts (27). This increase in PDE4 results in augmented degradation of intracellular cAMP, and by this pathway might impair nTreg/preTreg cell-mediated suppression. Therefore, an inhibition of PDE4 should lead to an improved suppressive capacity of nTreg/preTreg cells. Actually, we show that inhibition of PDE4 using two different inhibitors, rolipram and piclamilast, strongly enhanced the suppressive capacity of preTreg cells on Th2 cells. Certainly, PDE4 inhibitors will not only act on nTreg cells but also on Th2 cells leading to increased cAMP concentrations in suppressed Th2 cells. This assumption was corroborated by our finding that Th2 cells contain synergistically enhanced concentrations of cAMP after coculture with nTreg cells and preTreg cells in the presence of rolipram (see Fig. 2). Thus, inhibition of PDE4 leads to a strongly improved nTreg cell-mediated suppression, due to reduced cAMP degradation in Th2 cells.

PDEs represent a promising target using PDE inhibitors for the treatment of autoimmune and allergic diseases, among them psoriasis and allergic asthma. Obviously, they affect a multitude of different cell types leading to profoundly elevated cAMP concentrations that subsequently give rise to a general immune suppressive milieu. In this context, Hammad et al. (28) showed that the capacity of dendritic cells to induce regulatory T cell responses is related to intracellular cAMP levels in dendritic cells. Furthermore, in models of systemic sensitization treatment with PDE4 inhibitors was effective in decreasing the development of AHR and airway inflammation (14). However, the conditions used in these models did not allow dissecting the influence of PDE4 inhibition on nTreg cell-mediated suppression. Furthermore, because virtually all effector cell types on site of inflammation are affected, clear-cut results regarding the role of nTreg cells in such a scenario as target for PDE4 inhibitors could not be expected. Therefore, we used a protocol of allergen-specific Th2 cell transfer to T and B cell-deficient mice and consecutive airway challenges (29) to assess the potency of PDE4 inhibition for nTreg cell-mediated suppression in vivo. In general, this model results in a considerably strong inflammatory response in the lung, reflected by ~80% of cells in the BAL being eosinophils. Treatment of Th2 cells in vitro solely with rolipram had no significant effect on their cytokine production and proliferation (see Fig. 1). Similarly, inhibition of PDE4 during allergen exposure of mice which solely received Th2 cells did not diminish the development of AHR and airway inflammation. This was also the case even when using high doses of PDE4 inhibitor (1 mg/kg). These findings are different as compared with the effect of PDE4 inhibitors observed in other models of allergic airway disease where treatment with PDE4 inhibitors was effective in decreasing the development of AHR and airway inflammation (11–14). All these studies were performed in wild-type animals and thus in the presence of nTreg cells. On the basis of the present data, it can therefore be assumed that the observed

effects of PDE4 inhibitors in those models could have been, at least partially, mediated by nTreg cells.

To assess the suppressive capacity of preTreg cells, which have been shown to efficiently suppress Th2 response in vitro (15) these cells were transferred before Th2 cells. However, transfer of these cells had no significant effect on AHR similarly to another report (7). With respect to tissue inflammation and goblet cell metaplasia, preTreg cells could moderately decrease these features, suggesting in principle inhibitory effects of transferred preTreg cells as has been described in other studies (5, 9). Most effective in suppression of all characteristics of allergic airway disease, including AHR, was the combined treatment with preTreg cells and the PDE4 inhibitors rolipram and piclamilast. Their effect was not mediated by a de novo generation or expansion of preTreg cells, because it did not influence the frequency of CD4⁺CD25⁺Foxp3⁺ (preTreg) cells in the BAL fluid (see supplemental Figure S3). The underlying mechanism of action is at least partially based on the considerably increased concentration of cAMP in suppressed Th2 cells in vivo (see Fig. 6B). This furthermore suggests that PDE inhibition increases the regulatory capacity of preTreg cells and/or the susceptibility of Th2 cells to preTreg cell-mediated suppression. A similar effect could be observed in a therapeutic model of airway disease (see Fig. 7 and supplemental Fig. S5). In this secondary model of allergic airway disease transfer of nTreg cells in combination with PDE4-inhibitor, treatment was most effective in reduction of AHR and airway inflammation. In addition, it should be mentioned, that the dose of rolipram used in our model had been demonstrated to have moderate effects on AHR and inflammation in previous studies (14), whereas in the present study the combination of nTreg cells and PDE4 inhibition nearly completely suppressed AHR. Finally, it was shown very recently, that the application solely of nTreg cells could not inhibit established airway hyperreactivity (30). Thus, the combined approach described herein is also effective in a therapeutic setting pointing to the therapeutic potency of PDE4 inhibitors for the treatment of allergic diseases.

Potential innovative treatment of allergic asthma could involve the application of ex vivo-generated or modified nTreg cells to patients (31), and preclinical studies in models of allergic airway disease have demonstrated effectiveness of this approach (5). Despite some improvement in expansion of human nTreg cells in vitro (32, 33), the number of cells that are available is limited and the applicability for large cohorts of patients questionable. Therefore, additional pharmacotherapies that support the effectiveness of nTreg cells and might help to reduce the necessary cell number are desirable. In the present study, we demonstrate that inhibition of PDE4 decisively augments the inhibitory capacity of transferred nTreg cells in the setting of Th2 cell-induced allergic airway disease. Furthermore, preTreg cell transfer in combination with PDE4 inhibition was also effective after allergen re-exposure in previously sensitized mice, a model that certainly resembles more closely human exposure history. These findings suggest that additional pharmacological therapy could improve the effectiveness of nTreg cell application in suppressing allergic airway disease and might help to decrease the number of required nTreg cells in a therapeutic setting. Different approaches have demonstrated an effect of corticosteroids (34) or vitamin D3 (35) to induce IL-10 producing CD4⁺ T cells also termed iTreg cells. In this context, it is noteworthy that corticosteroids and PDE4 inhibitors have comparable effectiveness in the reduction of late airway response following allergen challenge in patients with allergic asthma, suggesting similar anti-inflammatory capacity (36, 37). Thus, in this scenario, pharmacotherapy with PDE4 inhibitors will strongly increase the suppressive capacity of nTreg cells and simultaneous

treatment with corticosteroids will recruit additional iTreg cells. This novel approach for the treatment of allergic asthma could also have the advantage that the efficacious concentrations of both agents can be reduced so that undesirable side effects can be prevented.

In summary, cAMP-dependent suppression of Th2 cells by nTreg cells is synergistically enhanced by blocking cAMP degradation in Th2 cells using the PDE4 inhibitor rolipram. The confirmation of this result in two different in vivo models of allergic airway disease, prophylactic and therapeutic as well, indicates that the selective improvement of the suppressive potency of nTreg cells is a promising intervention strategy to suppress immune responses unwanted in allergic diseases.

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Disclosures

The authors have no financial conflict of interest.

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Supplemental Figure S1: Scheme of the experimental protocol.

Rag2^{-/-} mice received either PBS or 2x10⁶ preTreg cells on day 0 cells by intravenous injection (i.v.). On day seven, 2x10⁶ Th2 cells were applied by i.v. injection and received six challenges with OVA on days 8 to 13 by ultrasonic nebulization (OVA inhalation). 30 minutes before each OVA challenge mice were treated with either PBS or rolipram by i.p. injection. On day 14 airway reactivity was assessed and animals were sacrificed on the same day for the collection of broncho-alveolar lavage fluid, blood, and lung tissue.

Supplemental Figure S2: Schematic representation of the sensitization, challenge and treatment protocol.

Mice were sensitized intraperitoneally on days 0 and 14 with OVA (OVA i.p.), and received three (primary) challenges with OVA on days 28, 29, and 30 by ultrasonic nebulization (OVA inhalation). Six weeks after the last of the three primary OVA challenges, mice were exposed to 1% OVA for 20 min by nebulization (OVA challenge). Airway function was assessed at 48 h after 1% OVA provocation (secondary challenge). Mice received a single intravenous injection of preTreg cells 24 h before OVA provocation, or an intraperitoneal injection of rolipram 2 h before and 6 and 24 h after OVA challenge.

Supplemental Figure S3: Cell count in BAL fluid.

Panel A: Flow cytometry analysis of BAL fluid 24 h following the last airway challenge. FFC/SSC, CD4-staining and CD25 and intracellular FoxP3 staining are shown for one example from each group of mice which received Th2 cells (Th2), Th2 cells and rolipram (Th2 rolipram), Th2 cells and preTreg cells (Th2 preTreg), Th2 cells and preTreg cells and rolipram (Th2 preTreg rolipram).

Panel B displays absolute number of CD4⁺ T cells in BAL fluid and panel C the percentage of preTreg cells (CD4⁺CD25⁺FoxP3⁺) cells with regard to total CD4⁺ cells that migrated to the lung.

Supplemental Figure S4:

Airway responsiveness (R_L) was measured in mice 48 h following the secondary challenge. Sensitized and challenged animals (sens/chall, n=5) developed increased reactivity to inhaled MCh compared to challenged only mice (chall, n=5). Administration of either 1×10^6 (1×10^6 preTreg, n=5) or 2.5×10^6 pre Treg cells (2.5×10^6 preTreg, n=5) prior to secondary challenge had no effect on airway reactivity, whereas transfer of 5×10^6 pre Treg cells (5×10^6 preTreg, n=5) resulted in significant decrease in AHR. Mean $\pm$ SEM are given. * $p < 0.05$.

Supplemental Figure S5: PDE4 inhibitor piclamilast augments the suppressive potency of preTreg cells in a therapeutic model of allergic airway disease.

Airway responsiveness (panel A) and numbers of eosinophils in BAL fluid (panel B) were measured in mice 48 h after a secondary airway challenge. Mice which were sensitized and challenged (sens/chall, n=3) showed increased airway reactivity and numbers of eosinophils in BAL fluid compared to challenged only mice (chall, n=3), where no eosinophils were detectable. Transfer of 2.5×10^6 preTreg cells (preTreg, n=2) or treatment with piclamilast (piclamilast, n=3) of sensitized and challenged mice had little effect on airway reactivity and numbers of eosinophils. In contrast, transfer of preTreg cells in combination with rolipram (preTregs piclamilast, n=3) suppressed AHR and airway inflammation. Panel A means $\pm$ SEM are given. Panel B bar represents mean.

Figure S1:

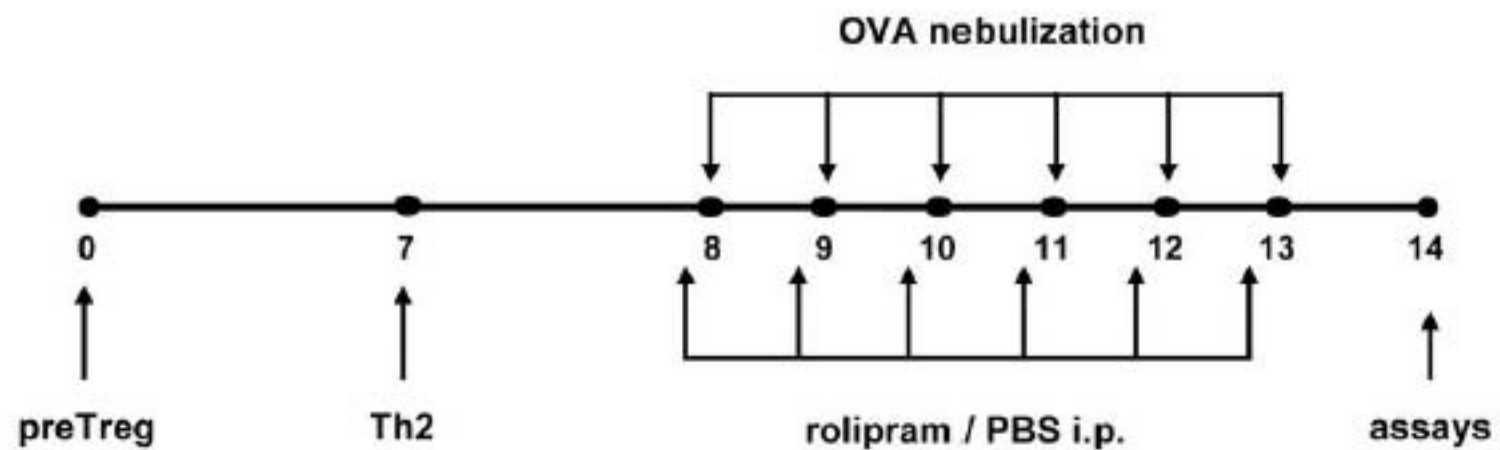


Figure S2:

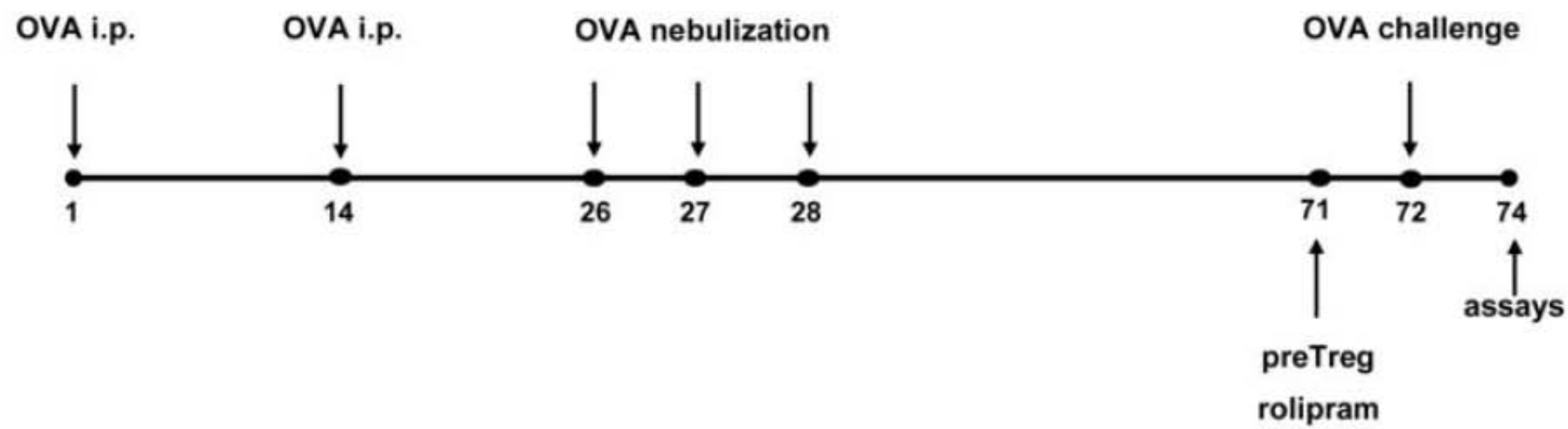


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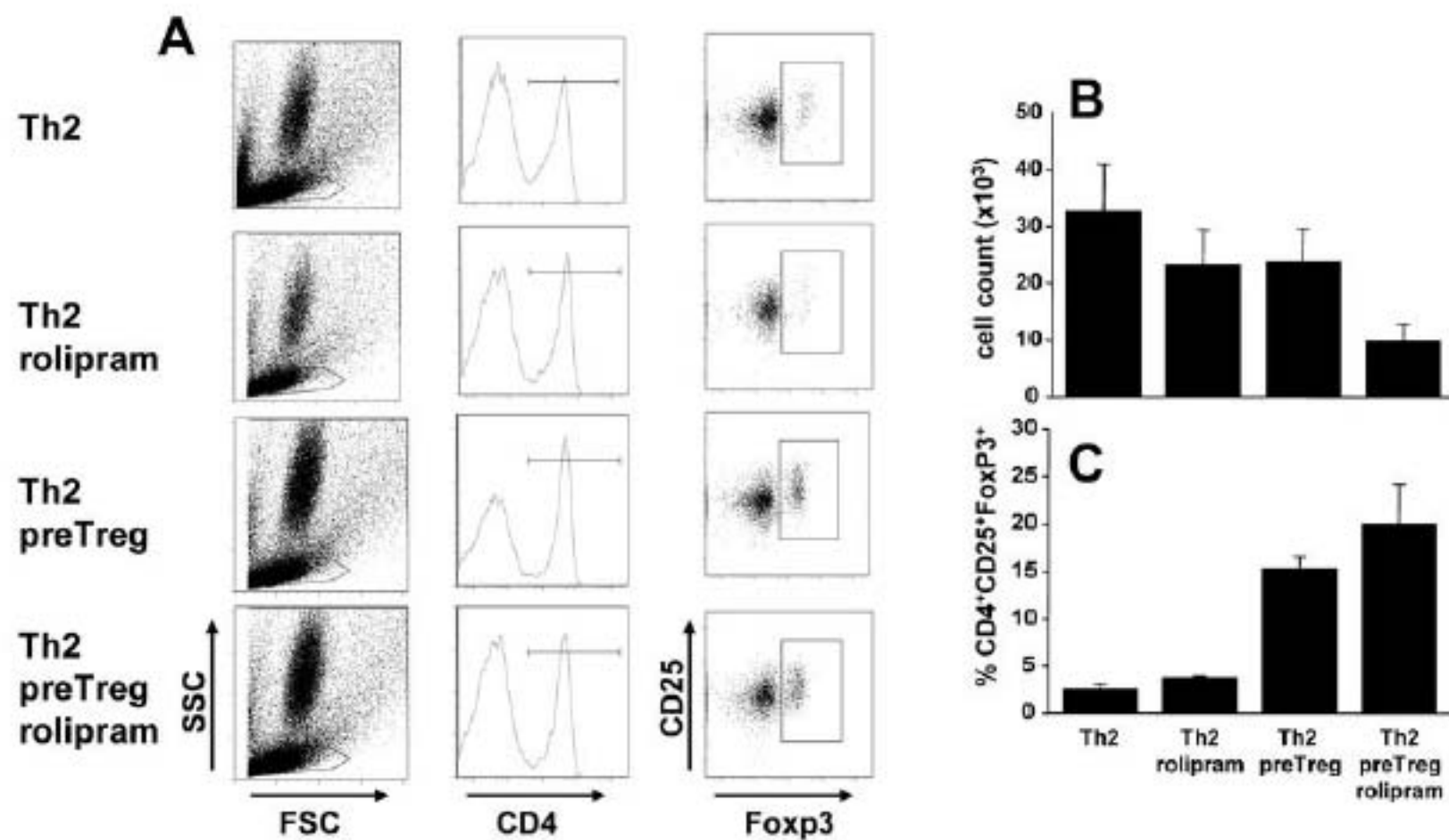


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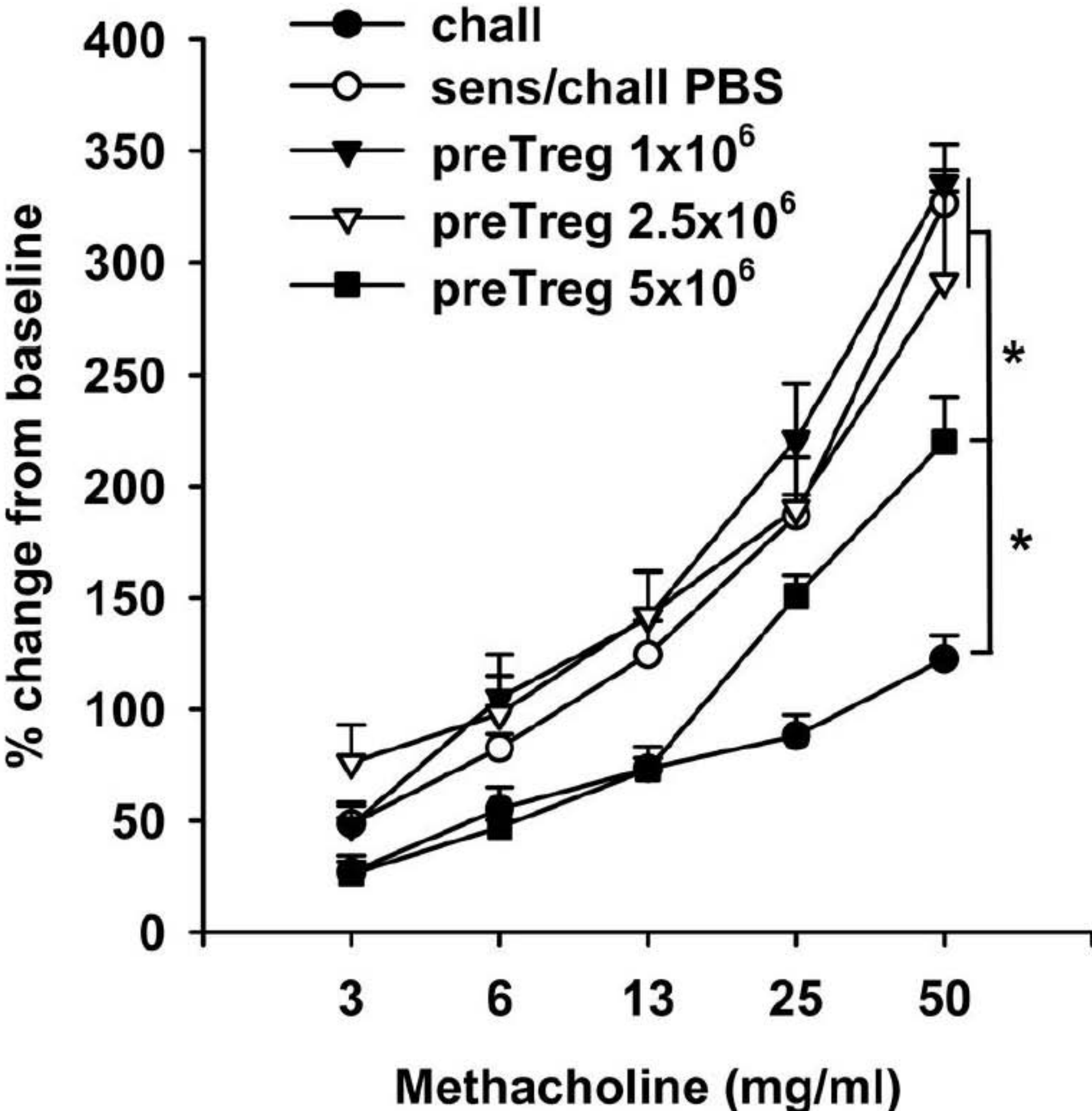


Figure S5:

