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Effect of inhaled and systemic glucocorticoid treatment on CD4$^+$ regulatory and effector T cells in a mouse model of allergic asthma

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ABSTRACT

To achieve a better understanding of mechanisms underlying the anti-asthmatic action of inhaled and systemic glucocorticoids (GCs) and to provide more data regarding the risk of a negative effect of inhaled GCs on CD4$^+$ T cells, a study was conducted on the effect of ciclesonide and methylprednisolone on CD4$^+$ effector (Teff), regulatory (Treg) and resting (Trest) T cells within respiratory and extra-respiratory tissues in a mouse model of allergic asthma. The study indicated that one, and possibly a key mechanism, underlying the anti-asthmatic action of inhaled and systemic GCs is the prevention of the activation and clonal expansion of CD4$^+$ Teff cells in the mediastinal lymph nodes (MLNs), which consequently prevents infiltration of the lungs with CD4$^+$ Teff cells. The beneficial effects of GCs in asthma treatment were not mediated through increased recruitment of Treg cells into the MLNs and lungs and/or local generation of Treg cells. The results demonstrated that inhaled and systemic GCs induced comparable depletion of normal CD4$^+$ Teff, Trest and Treg cells in the MLNs, head and neck lymph nodes and peripheral blood. Furthermore, inhaled, but not systemic GC therapy, led to the loss of these cells in the lungs. Thus, the study suggests that inhaled GC therapy may not be safer at all than systemic one with respect to the adverse effect on CD4$^+$ T cells present within and outside the respiratory tract. Moreover, administration of inhaled GCs can produce negative effects on lung-residing CD4$^+$ T cells.

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1. Introduction

CD4$^+$ T cells have a central role in the pathogenesis of asthma. Following antigen presentation by dendritic cells to recirculating naive T cells (Tn) in the mediastinal lymph nodes (MLNs), antigen-specific CD4$^+$ T cells are activated and differentiated into T-helper type 2 (Th2) effector type cells (Teff). These cells migrate to the lungs, where they orchestrate pulmonary immune responses and mediate the lung inflammation. More recently, regulatory T cells expressing the Foxp3$^+$-CD25$^+$CD4$^+$ phenotype (Treg cells) have been discovered as another pivotal subset of CD4$^+$ T cells involved in the pathogenesis of asthma. Many studies indicate that these suppressor cells are deficient or functionally impaired in people with asthma [1–3].

Inhaled and, in severe cases, systemic glucocorticoids (GCs) are the most effective anti-inflammatory drugs used to control disease symptoms and reverse bronchial hyperresponsiveness in asthma. The effect of inhaled GCs on CD4$^+$ T cells has been investigated in various aspects, but most of the research consisted of in vitro studies or relied on percentage analysis of these cells in peripheral blood (e.g. [4–7]). Noteworthy, a course of allergic asthma involves proliferation, redistribution and apoptosis of CD4$^+$ T cells in different immune compartments, that is the processes which do not occur in in vitro conditions and which cannot be captured and measured with the help of percentage analysis of T cells subsets in peripheral blood (PB). Generally, there is a significant gap in our knowledge in the scope of experimental studies which would evaluate and compare the effects of inhaled and systemic GC therapy on absolute counts, proliferation and apoptosis of effector and regulatory CD4$^+$ T cells within respiratory and extra-respiratory tissues in animal models with allergic asthma.

The principal hypothesis presumed that one of the mechanisms responsible for the therapeutic efficacy of inhaled and systemic GCs in the treatment of asthma is the inhibition of the activation and clonal expansion of CD4$^+$ Teff cells in the MLNs and/or inhibition of the recruitment of these cells into the lungs. This hypothesis is supported by the fact that the key mechanism of action of GCs is connected with the inhibition of the nuclear factor kappa B (NF-κB) [8], and it has been demonstrated that NF-κB plays critical roles in Th2 differentiation in allergic airway inflammation [9,10]. Bearing in mind the proapoptotic action of GCs [7], a possibility was also taken into consideration that the therapeutic action of these drugs in asthma patients can be related to the induction of apoptosis of CD4$^+$ Teff cells proliferating in the MLNs or infiltrating lungs.

Over the recent years, several reports have been published [4–7] suggesting that the complex mosaic of actions involved in the pro-

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inflammatory and immunosuppressive effects of GCs comprises such important elements as the induction and reinforcement of functions of Treg cells. In the light of the above, a hypothesis was put forward assuming that the anti-asthmatic effect of GCs could be associated with the expansion and/or accumulation of inducible Treg (iTreg) cells in the lungs and/or in the MLNs.

It is well documented that systemic GCs cause substantial systemic immunosuppression, which is linked to patients becoming more sensitive to various infections and this in turn leads to numerous contraindications for these drugs. This effect is produced inter alia by the negative influence on $CD4^+$ T cells [11,12]. Based on the available literature, it emerges that the issue of inhaled GCs affecting T cells – in terms of undesirable consequences – is scantily known. It is widely assumed that these drugs are incomparably safer than systemic GCs with respect to the immunosuppressive action. However, it should be underlined that we lack broad-scale research focusing on the following question: does inhaled GC therapy produce a negative effect on normal $CD4^+$ Teff and Treg cells within and outside the respiratory system, and if it does, is this effect comparable, weaker or stronger than the one effected by systemic GCs? These unresolved questions justify our decision to conduct research in this area.

Taking into account all the above considerations, the purpose of this study was to determine and compare: (a) involvement of $CD4^+$ T cells in the anti-asthmatic action of inhaled and systemic GCs, hence this part of the research was dedicated to the determination of the effect of these drugs on $CD4^+$ Teff and Treg cells engaged in the pathogenesis of asthma; (b) effect of inhaled and systemic GC therapy on $CD4^+$ T cells in the context of undesirable consequences, hence this part of the research aimed to determine the effect of these drugs on normal $CD4^+$ Teff, Treg and Trest (resting T) cells present within and outside the respiratory system. Ciclesonide (CIC) and methylprednisolone (MP) were used as a model for inhaled and systemic GCs, respectively. Both drugs have clinical application in treatment of allergic asthma.

2. Materials and methods

2.1. Animals and Ethics statement

The animals were housed and treated in accordance with the rules of the Local Ethics Commission for Animal Experiments in Olsztyn. All of the procedures were approved by the Local Ethics Commission (Ethic permission No. 35/2014/N). The experiments were carried out on 6-week-old Balb/c mice. Mice were bred and maintained under standard lab conditions [12/12 h light/dark cycle, controlled temperature (21 ± 2 °C) and humidity (55 ± 5%), and ad libitum access to food and water] in the Animal Facility of the Faculty of Veterinary Medicine, University of Warmia and Mazury in Olsztyn. Mice were euthanized by asphyxiation with CO_2.

2.2. Antigen immunization, airway challenge and treatment protocol

A schematic diagram showing the design the experiment is presented in Supplementary Fig. S1. Mice were divided into four groups, namely PBS (phosphate buffered saline) group (PBS-sensitized and -challenged mice, i.e. healthy mice/negative control), ovalbumin (OVA) group (OVA-sensitized and -challenged mice, i.e. mice with OVA-induced model of allergic asthma/positive control), OVA + CIC group (OVA-sensitized and -challenged mice treated with ciclesonide) and OVA + MP group (OVA-sensitized and -challenged mice treated with methylprednisolone sodium succinate; hereinafter referred to as methylprednisolone). Mice were sensitized on days 0 and 14 via intraperitoneal (i.p.) injection of 20 μg OVA (Grade V) emulsified in 2 mg aluminum hydroxide (AH) (both from Sigma-Aldrich, Schnelldorf, Germany) in total volume of 200 μl PBS. Mice were challenged intranasally (i.n.) with 100 μg of OVA in 50 μl of PBS. Mice in the negative control group (PBS group) received only AH in PBS (sensitization) or PBS alone (challenge).

Individual mice from OVA + CIC group were placed into an inhalation chamber and subsequently exposed to inhaled CIC (Alvesco, Takeda GmbH, Konstanz, Germany) at a dose of 160 μg [i.e. single dose (one puff)] once a day for 15 min. The animals from PBS, OVA and OVA + MP groups were placed in the inhalation chamber for the same length of time, but were not exposed to the action of CIC. MP (Solu-Medrol, Pfizer Manufacturing Belgium NV, Puurs, Belgium) was dissolved in saline solution and given intramuscularly (i.m.) at a dose 2 mg/kg/day, i.e. the dose which is used in the treatment of asthma. The animals from PBS, OVA and OVA + CIC groups were given i.m. saline solution. CIC, MP or placebo administration was started 48 h prior to i.n. challenge and continued for 4, 9 or 23 days, depending on the variant of the experiment; the drugs or placebo were given 3 h before challenge. For in vivo labeling of proliferating cells, mice were injected i.p. with 2 mg of 5-bromo-2′-deoxyuridine (BrdU; Sigma-Aldrich) every 24 h for 7 days (Supplementary Fig. S1). Samples of lungs for histopathological assays were taken at the second time point.

Apart from the above investigations, additional experiments were performed with the aim of determining the effect of CIC and MP on the analyzed cells in healthy animals, i.e. in non-OVA-immunized mice. To this aim, animals were divided into 3 groups: non-treated group (control group), CIC group (mice treated with ciclesonide) and MP group (mice treated with methylprednisolone). The drugs were administered every 24 h for 9 days in the same doses as described earlier. The role of a vehicle was achieved by placing the animals in an inhalation chamber for 15 min (control and MP groups) or injecting i.m. with saline solution (control and CIC groups). Mice were euthanized at the appropriate time points, as it is indicated in Supplementary Fig. S1.

2.3. Lung histology

Lung sections were prepared and stained as previously described [13]. Airway inflammation was quantified in the peribronchial region of 5 different medium-sized bronchi per slide on the basis of a scoring system previously described and used in similar studies [13,14]. The results were averaged and totaled for each mouse, and thereafter, the mean [± standard deviation (S.D.)] score per group was calculated. Epithelial thickness was measured at 4 sites for 5 different medium-sized bronchi per slide. All measurements were averaged, giving the mean (±S.D.) epithelial thickness per group.

2.4. Cell recovery

2.4.1. MLNs and HNLNs

MLNs and HNLNs were removed and subjected to dounce homogenization. The resulting cell suspensions were filtered through nitex fabric (Fairview Fabrics, Hercules, CA, USA), washed with Facs (fluorescence-activated cell-sorting) buffer [FB; Dulbecco's PBS devoid of Ca^{2+} and Mg^{2+} with 2% (v/v) heat-inactivated FBS (both from Sigma-Aldrich)], and centrifuged (300 ×g for 5 min. at 5 °C; the same parameters were used for all cell-washing procedures). Cells were re-suspended in FB, counted and stained for flow cytometric analysis.

2.4.2. Lung samples

Because the cell yield from lung samples of a single mouse is relatively poor, whole lungs obtained from three (assessment of apoptosis) or two (remaining experiments) animals were pooled into one sample. Lung lymphocytes were isolated by enzymatic digest (collagenase type IV, 50 U/ml; Sigma-Aldrich) and enriched by density gradient centrifugation (Percoll, Sigma-Aldrich) as previously described [13]. With respect to the evaluation of apoptosis, the collagenase step was omitted because the digestion process could cause false positive results.

2.4.3. PB

Blood was drawn from the inferior vena cava into heparinized or and EDTA coated tubes. Erythrocytes were removed as previously described [13]. The remaining cells were re-suspended in FB and stained for flow cytometric analysis. Routine CBC was performed using a hematology analyzer Advia 2120i (Siemens Healthcare Diagnostics, Erlangen, Germany).

2.5. Staining for flow cytometry analysis

2.5.1. Extracellular staining

Cells prepared as described above were stained for surface antigens with fluorochrome conjugated monoclonal antibodies (mAbs) specific to mouse CD4, CD8, CD25 and CD127 (all from BD Biosciences, San Jose, CA, USA), as demonstrated in Table 1; cells were stained for CD8 expression to distinguish $CD4^+CD8^-$ T cells from double positive, i.e. $CD4^+CD8^+$ T cells. After 45 min incubation, the cells were washed in 2 ml of FB. The staining combinations, properties of antibodies used in the experiments and evaluated parameters are summarized in Table 1.

2.5.2. Intracellular staining for Foxp3

Following surface staining, cells were washed, fixed, permeabilized (Mouse Foxp3 buffer set, BD Biosciences), and labeled with PE-conjugated rat anti-mouse Foxp3 mAb as previously described [13].

2.5.3. Staining for the evaluation of apoptosis

Cells stained for surface markers (i.e. for CD4, CD8, CD25 and CD127 molecules), were washed and labeled for apoptosis detection with Annexin V and 7-AAD as previously described [11].

2.5.4. Staining for the evaluation of proliferation

Cells were labeled for CD4 as described above and thereafter stained for incorporated BrdU according to the manufacturer's procedure (APC BrdU Flow Kit, BD Biosciences).

2.6. FACS acquisition and analysis

Flow cytometry analysis was performed using a FACSCanto II cytometer (BD Biosciences). The data were acquired by FACSDiva version 6.1.3 software (BD Biosciences) and analyzed by FlowJo software (Tree Star Inc., Stanford, USA). Absolute cell counts of lymphocyte subsets (i.e. number of cells from a particular subpopulation per μl of blood or per tissue sample) were calculated using the dual platform method, i.e. the absolute cell count was determined by calculating the data obtained from CBC (PB) or a cell counting chamber by the percentage of particular cell subsets. Supplementary Fig. S2 presents a gating strategy for flow cytometric data analysis and specifies how the absolute count was calculated for particular cell subsets.

2.7. Statistical analyses

Data were expressed as the mean (±S.D.) for 10 or 8 mice/sample per group. Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by the Bonferroni's post hoc test. Differences were deemed significant when the p values were <0.05.

3. Results

3.1. CIC and MP prevents OVA-induced allergic airway inflammation

All mice in OVA group developed signs characteristic for asthmatic inflammation (Fig. 1C). The influx of inflammatory cells occurred around the bronchi and bronchioles; the invading cells were composed mainly of eosinophils (Fig. 1C, H&E). Goblet cell hyperplasia and mucus hypersecretion were observed (Fig. 1C, PAS). In contrast, there was no airway inflammation in PBS, OVA + CIC and OVA + MP groups (Fig. 1C), with the exception of two mice from the MP-treated group which showed distinct signs of allergic inflammation.

The mean scores for OVA group were significantly increased compared to PBS, OVA + CIC and OVA + MP groups (Fig. 1A). The mean scores for OVA + CIC group did not differ significantly from OVA + MP group (Fig. 1A).

Quantitative assessment of the epithelial thickness demonstrated a significant increase with regard to this parameter in OVA group compared to PBS, OVA + CIC and OVA + MP groups (Fig. 1B). Epithelial thickness did not differ significantly between PBS, OVA + CIC and OVA + MP groups (Fig. 1B). No differences were found in the epithelial thickness between the steroid-treated groups (Fig. 1B).

3.2. In the MLNs, CIC and MP prevents OVA-induced increase in absolute counts of all studied $CD4^+$ T cell subsets and cause a depletion of $Foxp3^+$-$CD25^+CD4^+$ and $Foxp3^-CD25^+CD4^+$ T cells

In the MLNs, immunization with OVA induced an increase in absolute counts of all studied subsets, although this increase was transient in the case of $Foxp3^+CD25^+CD4^+$ (Fig. 2A) and $Foxp3^-CD25^+CD4^+$ (Fig. 2B) T cells, i.e. it occurred only at the second time point, whereas an increase in the count of $CD25^-CD4^+$ T cells (Fig. 2C) appeared at all time points. Administration of CIC and MP prevented the OVA-induced increase in absolute counts of $Foxp3^+CD25^+CD4^+$, $Foxp3^-$-$CD25^+CD4^+$ and $CD25^-CD4^+$ T cells in the MLNs. This is proven by the fact that the absolute counts of these cells from OVA + CIC and OVA + MP groups did not differ significantly from the values of this parameter obtained in PSB group, while being considerably lower than in OVA-immunized mice. In the animals treated with the drugs, a significant reduction was observed in the counts of $Foxp3^+CD25^+CD4^+$ (Fig. 2A) and $Foxp3^-CD25^+CD4^+$ (Fig. 2B) T cells at the third time point compared to the value of this parameter achieved in PBS group.

Table 1
The staining combinations, properties of antibodies used in the experiments and evaluated parameters.

Staining combinations	Clone	Isotype	Evaluated parameters
FITC/anti-mouse CD4	H129.19	IgG2a, κ	The absolute number of:
APC-Cy7/anti-mouse CD8a	53-6.7	IgG2a, κ	➔$Foxp3^+CD25^+CD4^+$ T cells
PE-Cy7/anti-mouse CD25	PC61	IgG1, λ	➔$Foxp3^-CD25^+CD4^+$ T cells
PE/anti-mouse Foxp3	MF23	IgG2b	➔$CD25^-CD4^+$ T cells
FITC/anti-mouse CD4	H129.19	IgG2a, κ	The percentage of early
APC-Cy7/anti-mouse CD8a	53-6.7	IgG2a, κ	apoptotic (Annexin $V^+7\text{-}AAD^-$)
PE-Cy7/anti-mouse CD25	PC61	IgG1, λ	cells within:
PE-CF594/anti-mouse CD127	SB/199	IgG2b, κ	➔ $CD127^{low/-}CD25^+CD4^+$ T cells
PE/Annexin V	-	-	➔$CD127^{high}CD25^+CD4^+$ T cells
7-AAD	-	-	➔$CD25^-CD4^+$ T cells
FITC/anti-mouse CD4	H129.19	IgG2a, κ	The absolute count of proliferating
APC/anti-BrdU	BU1/75	IgG2a	($BrdU^+$) $CD4^+$ T cells

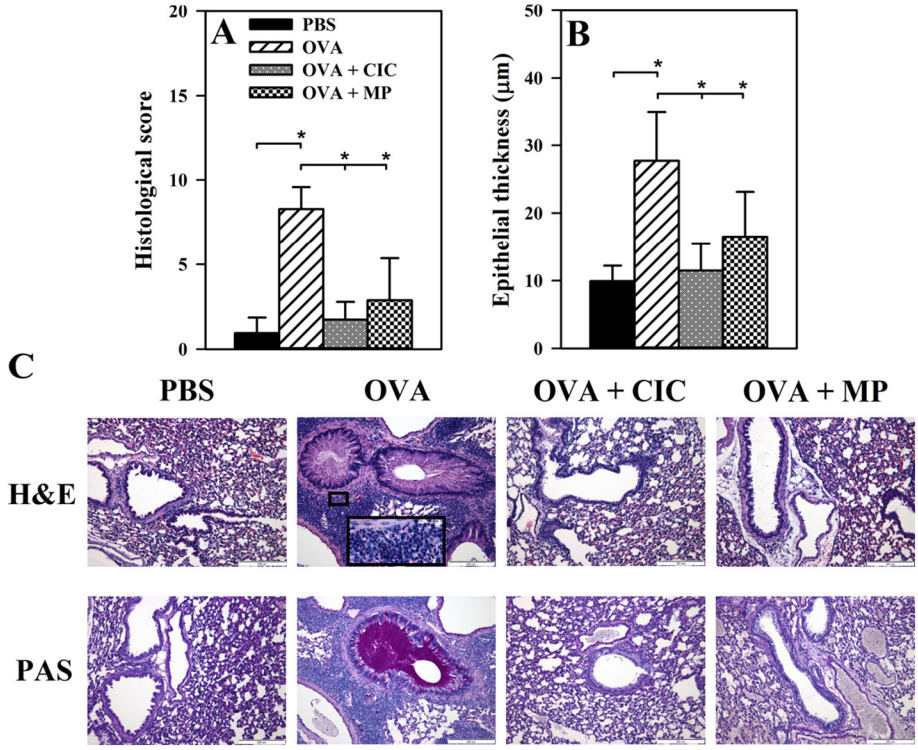

Fig. 1. Quantitative assessment of allergic airway inflammation and treatment efficacy. Mean histology scores of airway inflammation (A) and mean epithelial thickness (B). Results are expressed as the mean (±S.D.) of two independent experiments with 5 animals per group (n = 10 per group, $^{*}p < 0.001$). Examples of photomicrographs of tissue sections stained with haematoxilin and eosin (H&E) and periodic acid-Schiff (PAS) (C). Densely infiltrated eosinophils and lymphocytes (enlargement of the rectangle in the upper photograph) with mucous hypersecretion are visible in lung sections of ovalbumin-immunized mouse (OVA), while there are not histopathological abnormalities in sections of non-immunized mouse (PBS) and OVA-immunized mice treated with ciclesonide (OVA + CIC) or methylprednisolone (OVA + MP). OVA-sensitized mice were challenged with OVA for 7 days. The treatment with CIC and MP was started 48 h prior to OVA challenge and was continued daily for the next 7 days. One day after the last treatment and challenge, mice were sacrificed, and lung samples were individually collected. Magnification: 20-fold; 100-fold for the insert.

3.3. CIC and MP prevent OVA-induced increase in absolute counts of all studied CD4$^+$ T cell subsets in the lungs

The absolute numbers of Foxp3$^+$CD25$^+$CD4$^+$ (Fig. 3A), Foxp3$^-$CD25$^+$CD4$^+$ (Fig. 3B) and CD25$^-$CD4$^+$ (Fig. 3C) T cells were considerably increased in the lungs of mice from OVA group, and this increase was persistent as it appeared at both the second and the third time point. An increase in the count Foxp3$^+$CD25$^+$CD4$^+$ (Fig. 3A) T cells took place the earliest, having been demonstrated already at the first time point. In the groups treated with the drugs, the absolute number of Foxp3$^+$CD25$^+$CD4$^+$ (Fig. 3A), Foxp3$^-$CD25$^+$CD4$^+$ (Fig. 3B) and CD25$^-$CD4$^+$ T cells did not differ from counts of these cells in healthy animals (or was even lower than in healthy mice, as was shown for CD25$^-$CD4$^+$ T cells in OVA + CIC group at the last time point), while the values of these parameters were significantly lower compared with the OVA-immunized mice. Thus, the administration of CIC and MP prevented an OVA-induced increase in absolute counts of all studied subsets of CD4$^+$ T cells in the lungs.

3.4. Both immunization with OVA and co-administration of OVA and CIC or MP induce depletion of all studied CD4$^+$ T cell subsets in the HNHLs

It was observed that both at the second and at the third time point the absolute number of cells from all analyzed CD4$^+$ T cell subsets (except Foxp3$^-$CD25$^+$CD4$^+$ T cells in OVA group at the second time point) in the HNLN samples originating from mice from OVA group as well as OVA + CIC and OVA + MP groups was significantly lower than the corresponding values obtained in PBS group (Fig. 4).

3.5. Both immunization with OVA and co-administration of OVA and CIC or MP induce depletion of Foxp3$^+$CD25$^+$CD4$^+$ and CD25$^-$CD4$^+$ T cells in PB

A significant reduction was found for PB in the number of Foxp3$^+$CD25$^+$CD4$^+$ and CD25$^-$CD4$^+$ T cells, both in untreated OVA-immunized mice and in CIC- or MP-treated OVA-immunized mice (Fig. 5A and C). This reduction was persistent because it was documented at the second and third time point. It was also demonstrated that there was a transient depletion of Foxp3$^-$CD25$^+$CD4$^+$ T cells in mice of both OVA and OVA + CIC groups, recorded only at the second time point (Fig. 5B).

3.6. CIC, but not MP, induces the loss of all studied CD4$^+$ T cell subsets in the lungs of non-immunized mice & Administration of CIC and MP leads to depletion of all studied CD4$^+$ T cell subsets in the MLNs and HNLNs of non-immunized animals

The research showed that the absolute counts of Foxp3$^+$CD25$^+$CD4$^+$, Foxp3$^-$CD25$^+$CD4$^+$ and CD25$^-$CD4$^+$ T cells in lungs of mice exposed to the action of CIC was significantly lower than the corresponding control values (Fig. 6A, A′ and A″). In turn, the administration of MP did not affect significantly the number of these cells in lungs (Fig. 6A, A′ and A″). This is confirmed by the fact that the counts of Foxp3$^+$CD25$^+$CD4$^+$, Foxp3$^-$CD25$^+$CD4$^+$ and CD25$^-$CD4$^+$ T cells in MP treated animals did not differ significantly from control values but were significantly higher than in animals administered CIC (Fig. 6A, A′ and A″). The absolute counts of cells from all studied subpopulations were significantly lower in the MLNs (Fig. 6B, B′ and B″) and HNLNs (Fig. 6C, C′ and C″) originating from animals given either CIC or MP than in

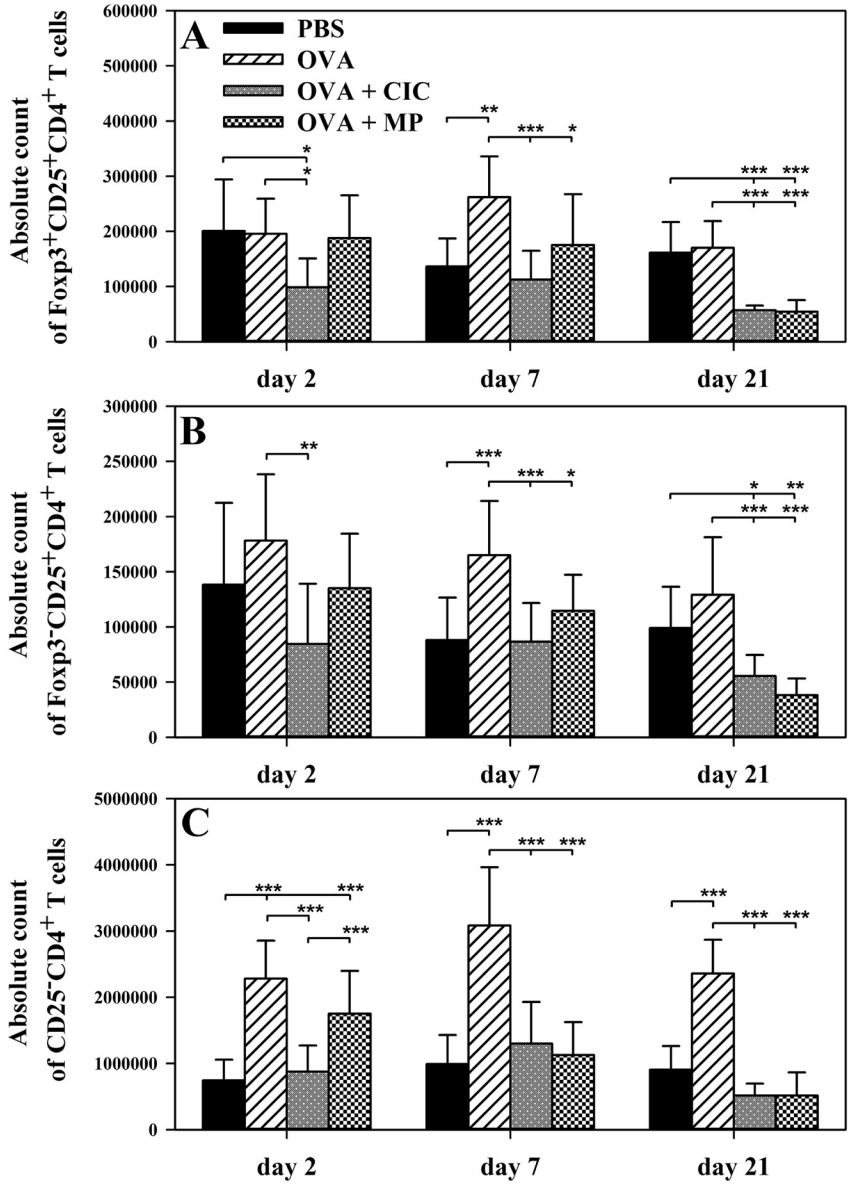

Fig. 2. Effect of inhaled and systemic glucocorticoid therapy on the number of $CD4^+$ T cells in the mediastinal lymph nodes (MLNs) of ovalbumin (OVA)-immunized mice. The absolute counts of $Foxp3^+CD25^+CD4^+$ (A) $Foxp3^-CD25^+CD4^+$ (B) and $CD25^-CD4^+$ (C) T cells were determined in the MLNs of non-immunized mice (PBS), OVA-immunized mice (OVA), ciclesonide-treated, OVA-immunized mice (OVA + CIC) and methylprednisolone-treated, OVA-immunized mice (OVA + MP). CIC and MP administration was started 48 h prior to OVA challenge. At the indicated number of days after challenge, mice were sacrificed, and MLN samples were individually collected. The absolute count represents the number of cells of each subset per MLN sample. Results are expressed as the mean (±S.D.) of two independent experiments with 5 mice per group (n = 10 per group, $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).

control animals. Another effect of the administration of either of the drugs was a loss $CD4^+$ T cells in PB. The absolute numbers of cells from all analyzed subsets were significantly lower in animals exposed to CIC (Fig. 6D, D′ and D″). In turn, the MP-treated mice were found to have significantly lower absolute counts of $Foxp3^+CD25^+CD4^+$ and $CD25^-CD4^+$ T cells, but not $Foxp3^-CD25^+CD4^+$, than the control values (Fig. 6D, D′ and D″). The loss of $CD25^-CD4^+$ T cells in CIC treated-mice was significantly larger than the one which occurred in mice exposed to MP (Fig. 6D″).

3.7. Administration of CIC and MP prevents OVA-induced proliferation of $CD4^+$ T cells in the MLNs

In order to explore the effect of CIC and MP on OVA-induced proliferative response of $CD4^+$ T cells, an experiment was conducted in which the absolute number of BrdU-incorporating $CD4^+$ T cells in the MLNs and lungs of untreated OVA-immunized mice and CIC- or MP-treated OVA-immunized mice was assessed. The research showed that OVA immunization led to a significant increase in the absolute number of $BrdU^+CD4^+$ T cells in the MLNs and lungs (Fig. 7A and B). The absolute number of $BrdU^+CD4^+$ T cells was significantly lower in the MLNs and lungs of CIC- or MP-treated OVA-immunized animals compared with the untreated OVA-immunized mice, but did not differ significantly from the value of this parameter achieved in non-immunized mice (Fig. 7A and B).

Considering the fact that the CIC- and MP-induced changes in counts of the examined cells could have been, to some extent, a result of the proapoptotic action of these drugs, a study was undertaken to resolve this question. As it is impossible, for certain methodological reasons, to determine apoptosis with respect to cells labeled

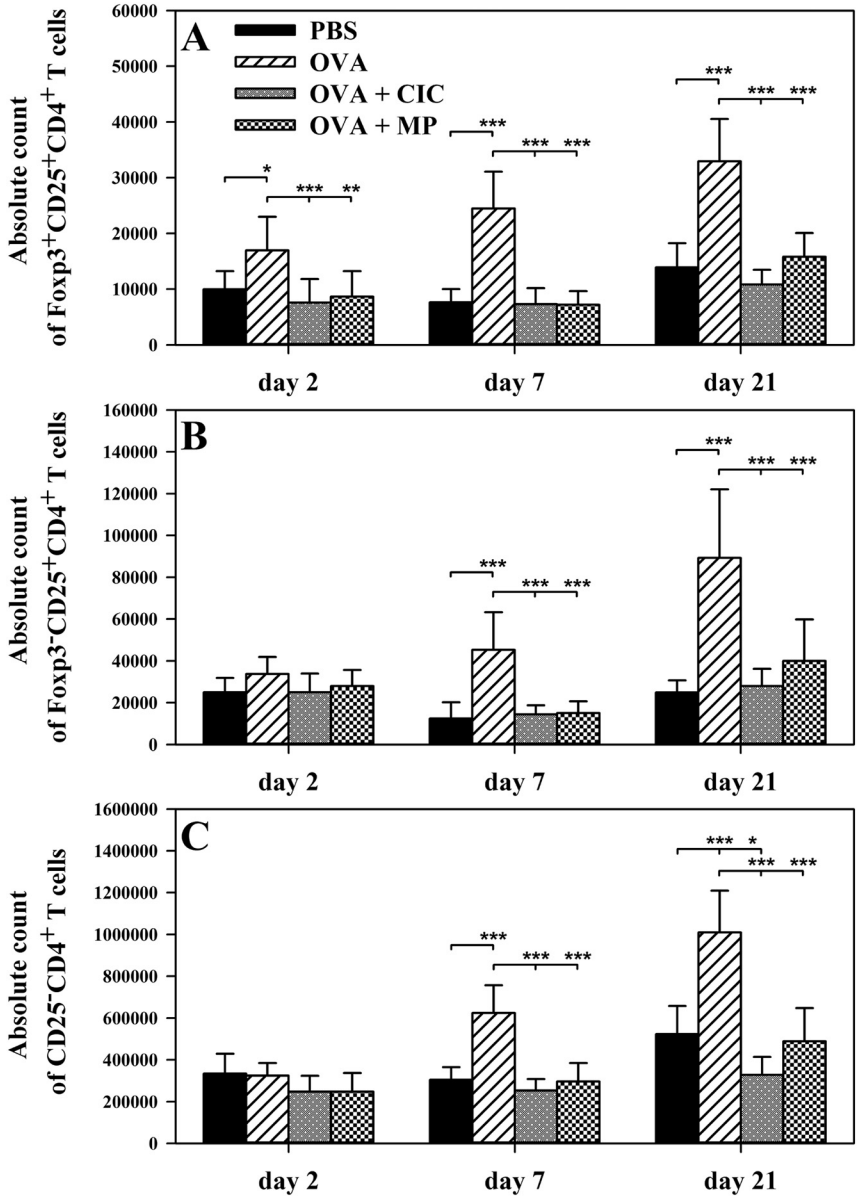

Fig. 3. Effect of inhaled and systemic glucocorticoid therapy on the number of $CD4^+$ T cells in the lungs of ovalbumin (OVA)-immunized mice. The absolute counts of $Foxp3^+CD25^+CD4^+$ (A) $Foxp3^-CD25^+CD4^+$ (B) and $CD25^-CD4^+$ (C) T cells were assessed in the lungs of non-immunized mice (PBS), ovalbumin-immunized mice (OVA), ciclesonide-treated, OVA-immunized mice (OVA + CIC) and methylprednisolone-treated, OVA-immunized mice (OVA + MP). CIC and MP administration was started 48 h prior to OVA challenge. At the indicated number of days after challenge, mice were sacrificed, and lung samples were collected. The absolute count represents the number of cells of each subset per lung sample pooled from two animals. Results are expressed as the mean (±S.D.) of two independent experiments involving 5 lung samples per group (n = 10 per group, $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).

towards Foxp3, alternative labeling towards CD127 (IL-7 receptor) was applied.

3.8. In the MLNs in mice with the model of asthma, but not in CIC- or MP-treated OVA-immunized mice, there is an increase in the percentage of early apoptotic cells among $CD127^{low/-}CD25^+CD4^+$ and $CD25^-CD4^+$ T cells

At the first time point, no effect was observed of either the immunization or the administered drugs on apoptosis of cells within any of the analyzed subsets. In turn, at the second time point, in OVA group, the percentage of early apoptotic cells among $CD127^{low/-}CD25^+$-$CD4^+$ and $CD25^-CD4^+$ T cells, but not among $CD127^+CD25^+CD4^+$, was significantly higher compared with the non-immunized animals, and with CIC- and MP-treated OVA-immunized mice (Supplementary Fig. S3).

3.9. There is elevated apoptosis of $CD127^{low/-}CD25^+CD4^+$ and $CD127^{high}CD25^+CD4^+$ T cells in lungs of both asthmatic and CIC-treated OVA-immunized mice

At the second, but not at the first time point, there was an increase in the apoptosis of $CD4^+$ T cells in lungs of both untreated OVA-immunized mice and CIC-treated OVA-immunized mice, whereas the intensity of this process in the MP group did not differ significantly from the values obtained in PBS group (Fig. 8). Our study demonstrated that the percentage of early apoptotic cells within $CD127^{low/-}CD25^+CD4^+$ and $CD127^{high}CD25^+CD4^+$ T cells was significantly higher in both OVA-immunized mice and OVA and CIC-treated mice than in non-immunized animals (Fig. 8A and B). With respect to $CD127^{low/-}CD25^+$-$CD4^+$ T cells, the percentage of early apoptotic cells was significantly higher in OVA-group than in OVA + CIC group (Fig. 8A). It was observed

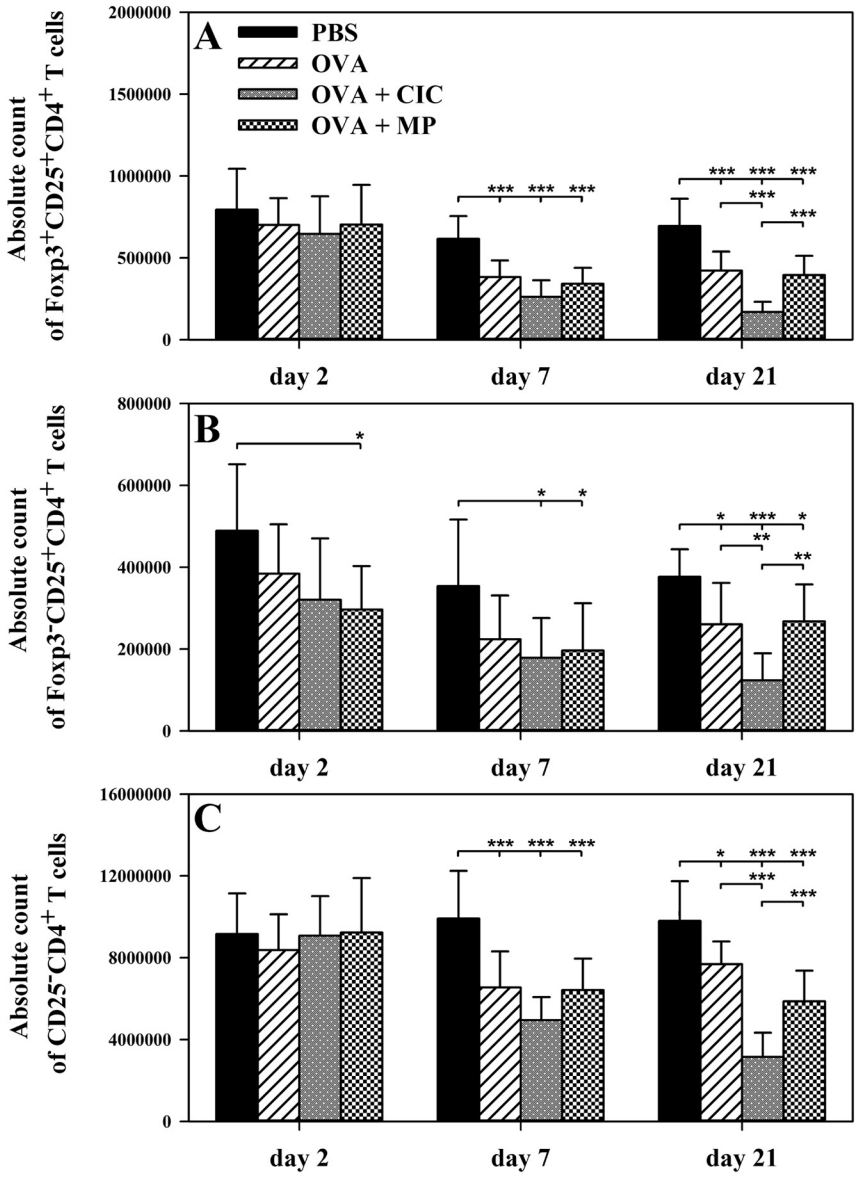

Fig. 4. Effect of inhaled and systemic glucocorticoid therapy on the number of $CD4^+$ T cells in the head and neck lymph nodes (HNLNs) of ovalbumin (OVA)-immunized mice. The absolute counts of $Foxp3^+CD25^+CD4^+$ (A) $Foxp3^-CD25^+CD4^+$ (B) and $CD25^-CD4^+$ (C) T cells were determined in the HNLNs of non-immunized mice (PBS), ovalbumin-immunized mice (OVA), ciclesonide-treated, OVA-immunized mice (OVA + CIC) and methylprednisolone-treated, OVA-immunized mice (OVA + MP). CIC and MP administration was started 48 h prior to OVA challenge. At the indicated number of days after challenge, mice were sacrificed, and HNLN samples were individually collected. The absolute count represents the number of cells of each subset per HNLN sample. Results are expressed as the mean (±S.D.) of two independent experiments with 5 mice per group (n = 10 per group, $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).

that the percentage of early apoptotic cells within $CD25^-CD4^+$ T cells was significantly higher in mice exposed to CIC than in non-immunized mice (Fig. 8C). No differences were determined in respect of the percentage of early apoptotic cells within $CD127^{low/-}CD25^+CD4^+$, $CD127^{high}CD25^+CD4^+$ and $CD25^-CD4^+$ T cells between non-immunized mice and MP-treated, OVA-immunized mice (Fig. 8A–C), although it was demonstrated that the value of this parameter was significantly higher in CIC group than in MP group (Fig. 8A–C).

3.10. Administration of CIC and MP leads to elevated apoptosis among all studied $CD4^+$ T cell subsets in the HNLNs

At the second time point, but not at the first one, it was found that the percentage of early apoptotic cells among $CD127^{low/-}CD25^+CD4^+$, $CD127^{high}CD25^+CD4^+$ and $CD25^-CD4^+$ T cells was significantly higher in CIC- and MP-treated OVA-immunized mice than in non-immunized ones (Fig. 9A–C). Likewise, compared to untreated OVA-immunized mice, GC-treated mice were detected to present a significant increase in the percentage of early apoptotic cells within $CD127^{low/-}CD25^+CD4^+$ and $CD25^-CD4^+$ and $CD127^{high}CD25^+CD4^+$ ($p < 0.05$) T cells (Fig. 9A–C). No differences were demonstrated in terms of the percentage of early apoptotic cells within $CD127^{low/-}CD25^+CD4^+$, $CD127^{high}CD25^+CD4^+$ and $CD25^-CD4^+$ T cells of PB between any of the groups of mice used in our experiments (Supplementary Fig. S4).

4. Discussion

4.1. Involvement of $CD4^+$ T cells in the anti-asthmatic action of GCs

Foxp3 currently represents the most specific marker used to distinguish Treg cells ($Foxp3^+CD25^+CD4^+$) from their thymic progenitors and activated Teff cells ($Foxp3^-CD25^+CD4^+$); the $CD25^-CD4^+$ phenotype represents Trest cells, i.e. Tn and memory T cells [15]. We found that the number of Treg cells in the lungs and MLNs of CIC- or MP-

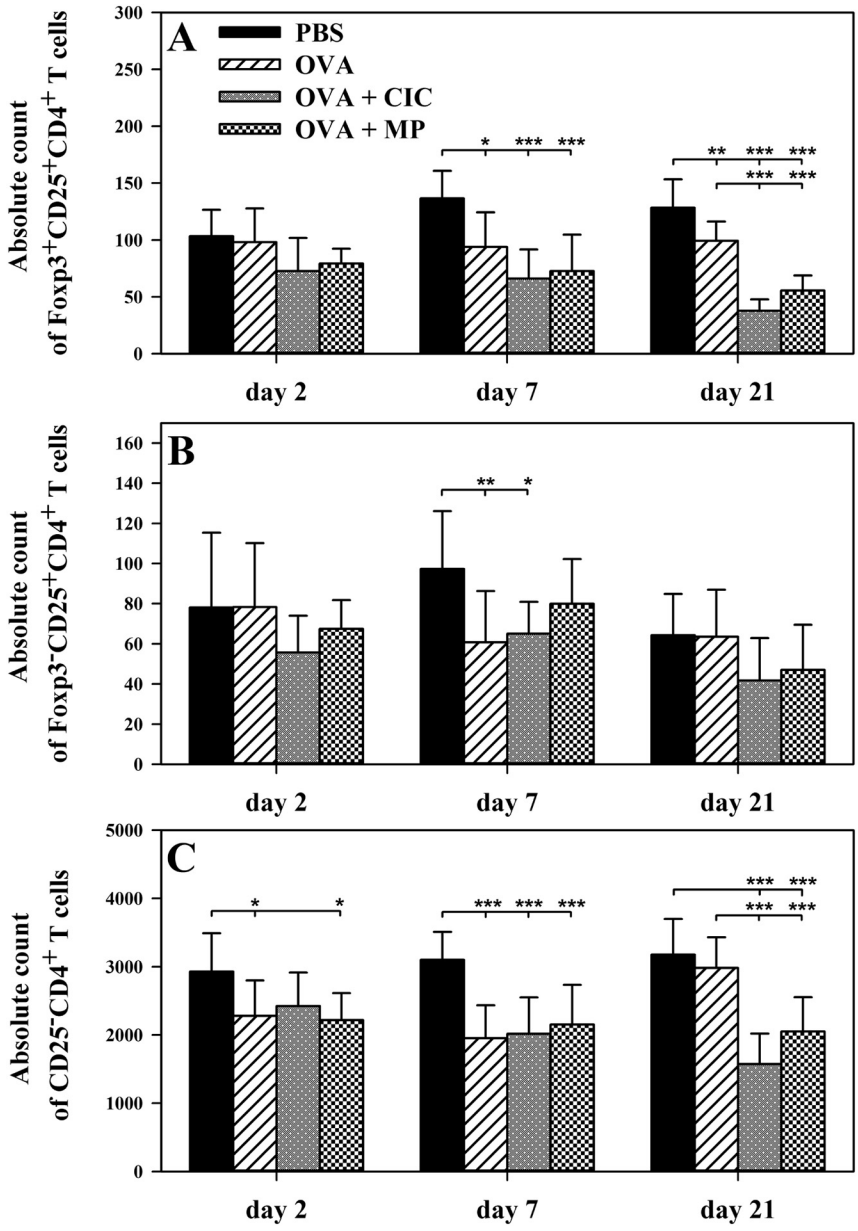

Fig. 5. Effect of inhaled and systemic glucocorticoid therapy on the number of $CD4^+$ T cells in peripheral blood (PB) of ovalbumin (OVA)-immunized mice. The absolute counts of $Foxp3^+CD25^+CD4^+$ (A) $Foxp3^-CD25^+CD4^+$ (B) and $CD25^-CD4^+$ (C) T cells were assessed in PB of non-immunized mice (PBS), ovalbumin-immunized mice (OVA), ciclesonide-treated, OVA-immunized mice (OVA + CIC) and methylprednisolone-treated, OVA-immunized mice (OVA + MP). CIC and MP administration was started 48 h prior to OVA challenge. At the indicated number of days after challenge, mice were sacrificed, and PB samples were individually collected. The absolute count represents the number of cells of each subset per μl of blood. Results are expressed as the mean (±S.D.) of two independent experiments with 5 mice per group (n = 10 per group, $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).

treated OVA-immunized mice did not differ significantly from that in the non-immunized animals. Moreover, the number of Treg cells in the lungs and MLNs of untreated OVA-immunized mice was significantly increased. This coincides with the results of the authors' latest study [13] or the one reported by Olsen et al. [16], in which such an effect was also revealed. Thus, our results indicate that the anti-asthmatic action of GCs is not mediated through: (a) increased recruitment of Treg cells into the lung and/or MLNs, (b) local generation of iTreg cells in the lungs and/or MLNs.

The present study showed a considerable increase in the number and proliferation of $CD4^+$ T cells in the MLNs in mice with asthma model. Moreover, the count of $BrdU^+$ $CD4^+$ T cells in the lungs also increased in these animals. All of these effects were fully prevented by the treatment with inhaled and systemic GCs. It can be concluded that one of the mechanisms, and most probably a key one, responsible for the anti-asthmatic effect of inhaled and systemic GCs is the prevention of the activation and clonal expansion of $CD4^+$ Teff cells in the MLNs.

The application of inhaled as well as systemic GCs prevented infiltration of the lungs with activated $CD4^+$ Teff cells, although most evidently this effect was not a consequence of the direct impact of the drugs on lymphocyte trafficking and homing into the lungs, but was an indirect result. As the administration of the drugs prevented the OVA-induced clonal expansion of $CD4^+$ Teff cells in the MLNs, this meant that no $CD4^+$ Teff cells were generated that would be available for recruitment into the lungs. In the light of this conclusion, the hypothesis claiming that one of the anti-asthmatic mechanisms of GCs could be the proapoptotic influence of the drugs on $CD4^+$ Teff cells seems to be false. Although it was found that CIC increased the apoptosis of all analyzed subsets of $CD4^+$ T cells in the lungs, it was not demonstrated that the CIC-treated mice presented any considerable infiltration of the lungs by

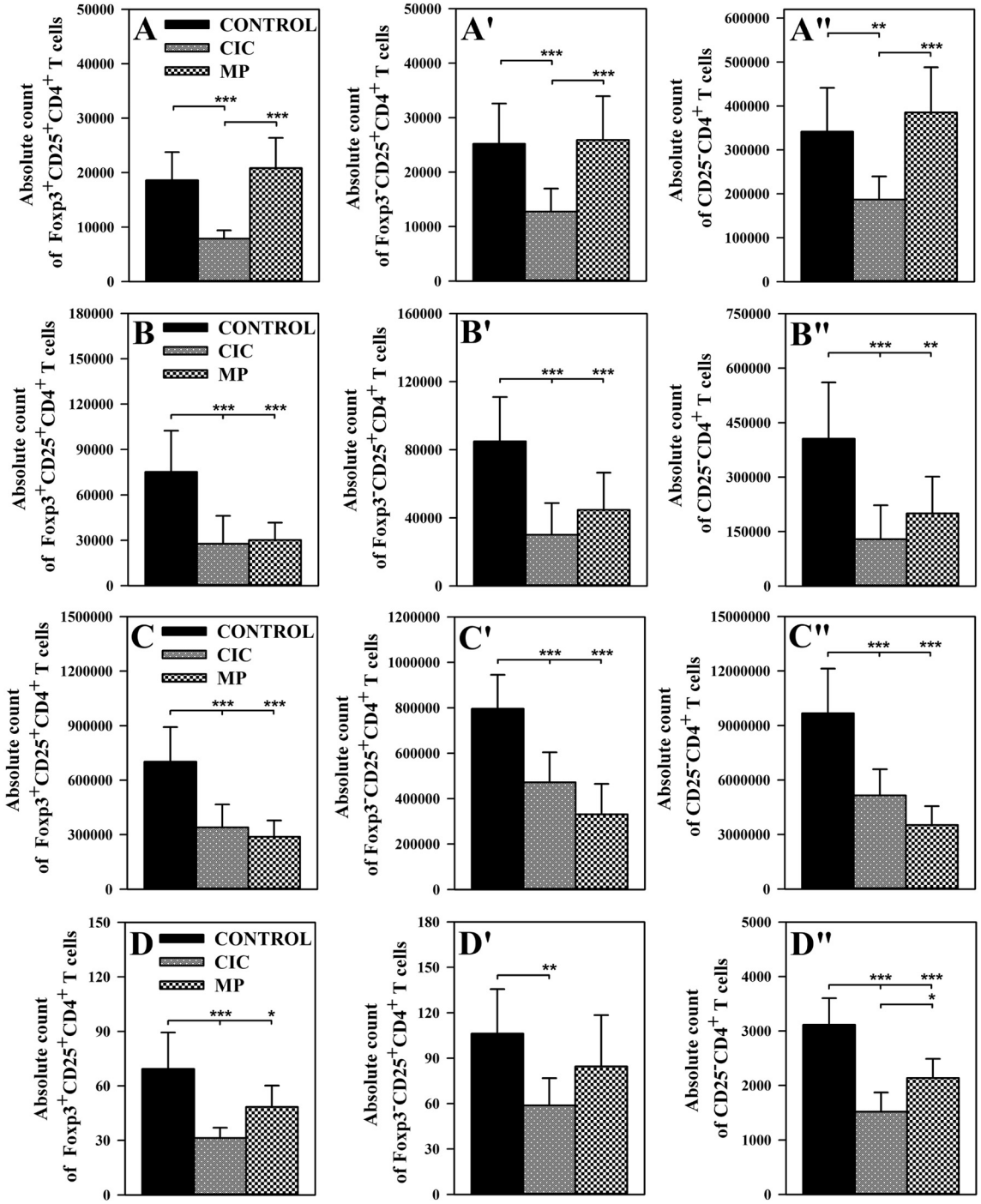

Fig. 6. Effect of inhaled and systemic glucocorticoid treatment on the number of $CD4^+$ T cells in the lungs, mediastinal lymph nodes (MLNs), head and neck lymph nodes (HNLNs) and peripheral blood (PB) of non-immunized mice. The absolute counts of $Foxp3^+CD25^+CD4^+$ (A, B, C and D) $Foxp3^-CD25^+CD4^+$ (A′, B′, C′ and D′) and $CD25^-CD4^+$ (A″, B″, C″ and D″) T cells were assessed in the lungs (A, A′ and A″), MLNs (B, B′ and B″), HNLNs (C, C′ and C″) and PB (D, D′ and D″) of non-treated mice (CONTROL) and ciclesonide-(CIC) or methylprednisolone-treated mice (MP). CIC and MP were administered once daily for 9 days, and 24 h after the last dose, mice were sacrificed, and lung samples were collected. The absolute count represents the number of cells of each subset per lung (pooled from two animals), MLN, HNLN and PB sample. Results are expressed as the mean (±S.D.) of two independent experiments, each involving 5 lung samples or 5 mice (MLNs, HNLNs and PB) per group (n = 10 per group, $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).

these cells. Hence, the discussed effect should rather be interpreted as the proapoptotic impact of CIC on naturally occurring in lungs $CD4^+$ T cells, and not on lung infiltrating pro-inflammatory $CD4^+$ T cells.

4.2. Evaluation of the effect of inhaled and systemic GC therapy on $CD4^+$ lymphocytes in the context of undesirable effects

Administration of CIC and MP in OVA-immunized and non-immunized animals caused depletion of studied cells in MLNs. In lungs of non-immunized mice, exposure to CIC but not to MP, caused losses in all analyzed $CD4^+$ T cell subsets. Moreover, there was a loss of $CD4^+$ Trest cells in the lungs of CIC-treated OVA-immunized mice after prolonged administration of the drug. The results justify the conclusion that both inhaled and systemic GC therapy can lead to some loss in MLN-residing regulatory and effector $CD4^+$ T cells. The risk of such an effect occurring in lungs is most evidently related to the administration of inhaled GC therapy. It should be noted that CIC and MP induced considerable losses of cells in all analyzed cell subsets in the MLNs of non-

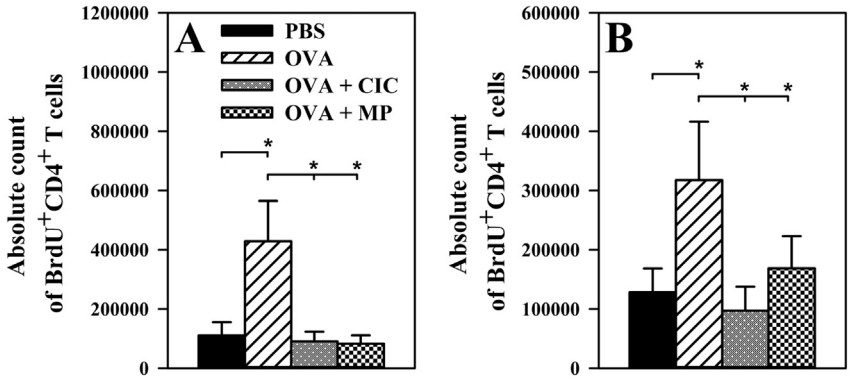

Fig. 7. Effect of inhaled and systemic glucocorticoid therapy on proliferation of $CD4^+$ T cells in in the mediastinal lymph nodes (MLNs) (A) and lungs (B) of ovalbumin (OVA)-immunized mice. The absolute counts of proliferating ($BrdU^+$) $CD4^+$ T cells were determined in the MLNs (A) and lungs (B) of non-immunized mice (PBS), ovalbumin-immunized mice (OVA), ciclesonide-treated, OVA-immunized mice (OVA + CIC) and methylprednisolone-treated, OVA-immunized mice (OVA + MP). OVA-sensitized mice were challenged with OVA for 7 days. The treatment with CIC and MP was started 48 h prior to OVA challenge and was continued daily for the next 7 days. Mice were injected i.p. with 5-bromo-2′-deoxyuridine (BrdU) every 24 h for 7 days. One day after the last treatment and challenge, mice were sacrificed, and MLN and lung samples were collected. The absolute count represents the number of $BrdU^+CD4^+$ T cells per MLN or lung sample; MLNs were collected from individual animals, whereas each lung sample consisted of cells pooled from two mice. Results are expressed as the mean (±S.D.) of two independent experiments with 5 mice (MLNs) or samples (lungs) per group (n = 10 per group, $^*p < 0.001$).

immunized mice, while CIC additionally caused a loss of these cells in the lungs, whereas no such effect was observed at the same time point in the OVA-immunized mice treated with the drugs. It is highly probable that OVA-immunized mice were less susceptible to the cytoreductive effect of GCs. It is supported by the fact that the systemic availability of fluticasone was substantially lower in patients with moderate-to-severe asthma than in healthy subjects [17].

Inhaled GC-induced depletion of $CD4^+$ T cells was not restrained to the lower respiratory tract, but occurred also outside it, i.e. in HNLNs and PB. Such effects could be expected following an application of MP, as the cytoreductive effect of systemic GCs had been described in literature, but our research results regarding CIC are quite surprising. It is worth noticing that inhaled GCs are subject to almost complete first-pass metabolism. Moreover, the fraction of des-CIC absorbed from the lungs after inhalation is highly protein bound (99%), resulting in minimal systemic exposure [18]. Thus, the results show that even such a small fraction of unbound des-CIC in the systemic circulation as achieved after an inhalatory application of CIC is sufficiently large to affect the extra-respiratory located lymphocytes.

A loss in the analyzed cells in the HNLNs and PB was also noted in untreated OVA-immunized mice. Noteworthy, the results of our study prove that the loss of $CD4^+$ T cells in GC-treated OVA-immunized mice was caused either completely or at least largely by the administered drugs rather than by immunization. This is evidenced by the occurrence of the same effect in non-immunized mice treated with CIC and MP.

The demonstration of CIC-induced depletion of $CD4^+$ T cells in HNLNs and PB is an extremely important and intriguing finding because it challenges the broadly accepted supposition that inhaled GCs do not have a destructive effect on T cells present outside the respiratory system, or at least that such an effect is much weaker than the one induced by systemic GCs. Our results strongly suggest that the use of CIC, and most probably other inhaled GCs, can cause a cytoreductive effect on $CD4^+$ T cells in extra-respiratory locations. The results of Olsen et al. [16] and are in accordance with ours, however, the only available study concerning the effect of CIC on $CD4^+$ T cells did not demonstrate that a 5-day-long application of the drug in asthma human subjects affected the absolute count of these cells in blood [19].

A question arises about the mechanism behind the GC-induced depletion of $CD4^+$ T cells revealed in our study. CIC, but not MP, induced the apoptosis of lung-residing $CD4^+$ T cells. Moreover, CIC and MP produced a proapoptotic effect towards all analyzed subsets of $CD4^+$ T cells in the HNLNs, although no such effect of the drugs on cells in the MLNs was detectable. Thus, the loss of studied cells was at least partly a consequence of their induced apoptosis. This conclusion is supported by results of Pace et al. [7], who demonstrated that in vitro exposure of the PBMCs to budesonide raised the apoptosis of $CD4^+$ T cells. It is highly doubtful whether the demonstrated loss of cells was due to the transfer of lymphocytes from PB to other tissues. Such redistribution would actually require the acquisition by lymphocytes of appropriate homing receptors, while there is no evidence suggesting that GCs increased the expression of these receptors.

The results of our research demonstrated that there were no differences, albeit with very few exceptions, between the inhaled and systemic GC therapy in terms of the analyzed parameters. Thus, surprisingly, these results strongly suggest that inhaled therapy may not be safer than the systemic administration of these drugs in respect of the unwanted effect on $CD4^+$ T cells present outside the respiratory tract. Moreover, it turns out that inhalatory administration of GCs can cause a significant risk of their negative effect on lung-residing $CD4^+$ T cells. These results strongly suggest that administration of inhaled GCs, similarly to systemic ones, can weaken the immune response towards various pathogens both within and outside the respiratory system. Interestingly, GCs decreased not only the counts of Teff and Trest cells but also those of Treg cells. This finding is somewhat paradoxical, because - as already mentioned by many researchers [4–7] - these drugs have been attributed the ability to generate iTreg cells.

5. Conclusions

The research reported in this article indicates that one of the mechanisms, and probably a key one, underlying the anti-asthmatic action of inhaled and systemic GCs is the prevention of the activation and clonal expansion of $CD4^+$ Teff cells in the MLNs, which in consequence prevents infiltration of the lungs with activated $CD4^+$ Teff cells. The beneficial effects of GCs in asthma treatment are not mediated through increased recruitment of Treg cells into the MLNs and lungs and/or local generation of iTreg cells. The results indicate that inhaled and systemic GCs may induce comparable losses of normal $CD4^+$ Teff, Trest and Treg cells in the MLNs, HNLNs and PB. Furthermore, inhaled but not systemic GC therapy can lead to depletion of these cells in the lungs. The cytoreductive effect of inhaled and systemic GCs can be associated, at least to some degree, with their proapoptotic action towards normal $CD4^+$ T cells. Hence, the current research results strongly suggest that inhaled GC therapy might not be safer after all than systemic one in respect of the undesirable effects on $CD4^+$ T cells present within and outside the respiratory

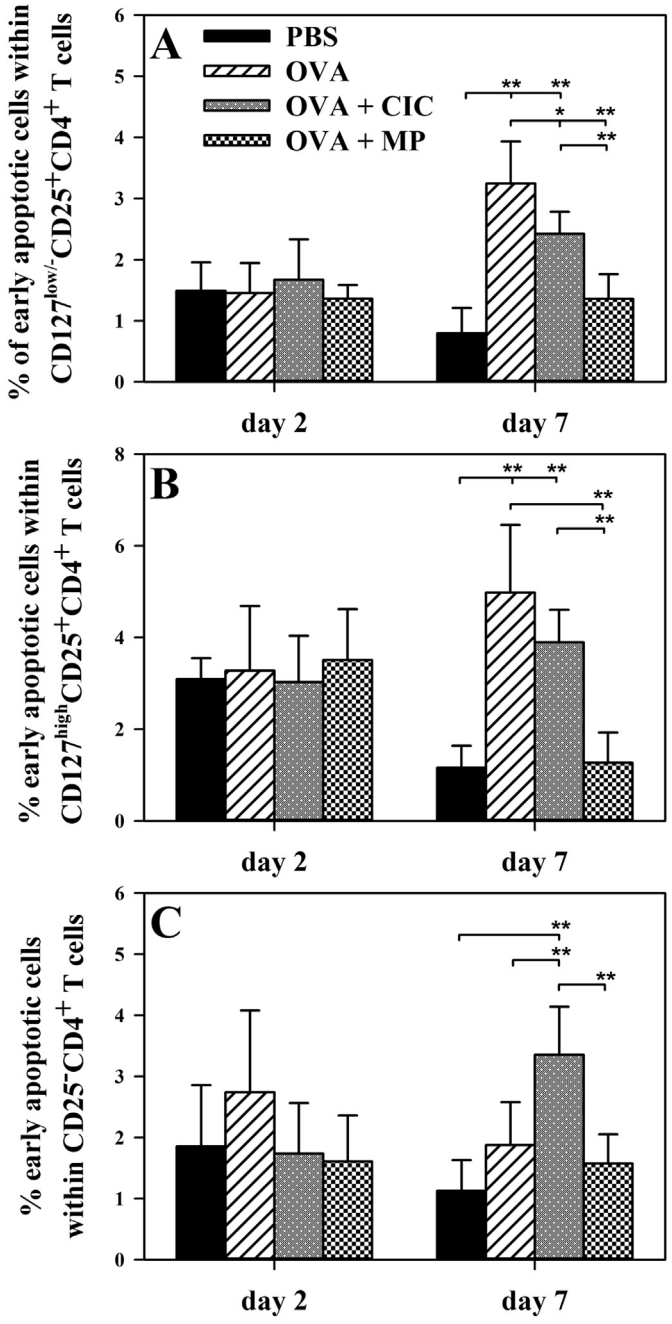

Fig. 8. Effect of inhaled and systemic glucocorticoid therapy on apoptosis of $CD4^+$ T cells in the lungs of ovalbumin (OVA)-immunized mice. The apoptosis of $CD127^{low/-}CD25^+CD4^+$ (A) $CD127^+CD25^+CD4^+$ (B) and $CD25^-CD4^+$ (C) T cells was assessed in the lungs of non-immunized mice (PBS), OVA-immunized mice, ciclesonide-treated, OVA-immunized mice (OVA + CIC) and methylprednisolone-treated, OVA-immunized mice (OVA + MP). CIC and MP administration was started 48 h prior to OVA challenge. At the indicated number of days after challenge, mice were sacrificed, and lung samples were individually collected. The results represent the percentage of $CD127^{low/-}CD25^+CD4^+$, $CD127^+CD25^+CD4^+$ and $CD25^-CD4^+$ T cells in early apoptosis (Annexin V^+7-AAD^- cells), and they are the mean (±S.D.) of two independent experiments involving 4 lung samples (each lung sample consisted of cells pooled from three mice) per group (n = 8 per group, $^*p < 0.05$, $^{**}p < 0.001$).

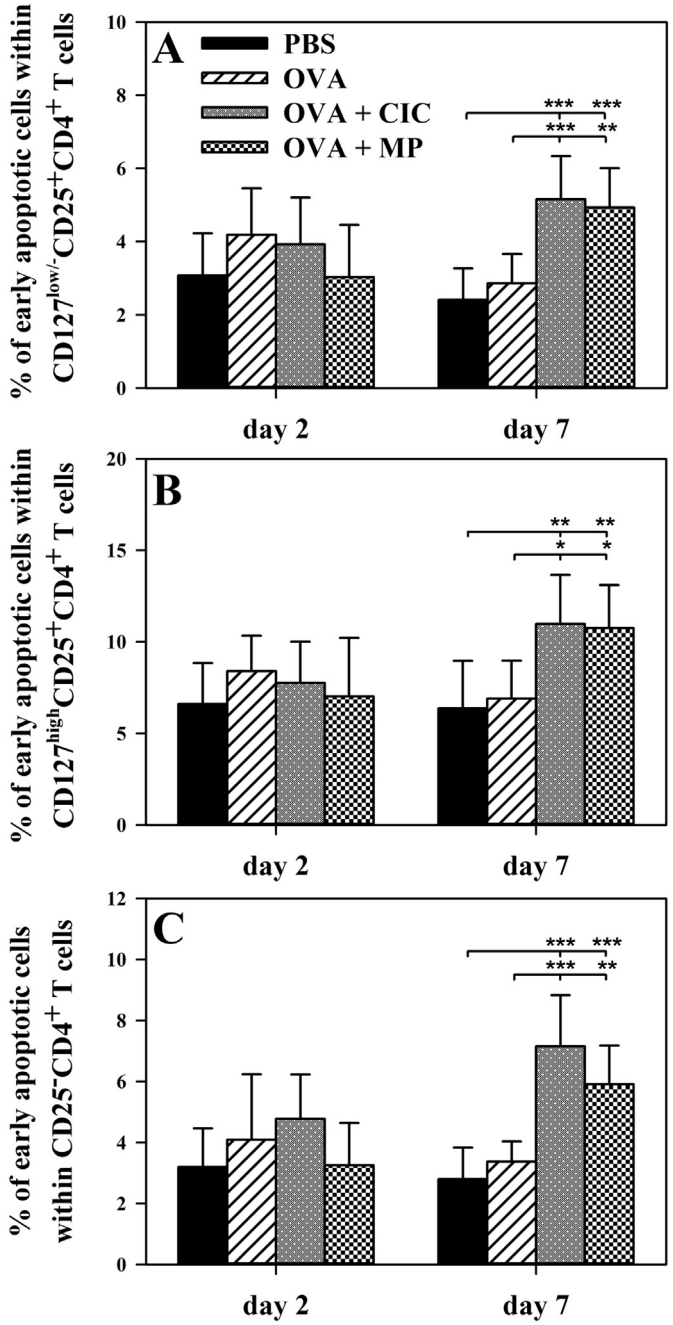

Fig. 9. Effect of inhaled and systemic glucocorticoid therapy on apoptosis of $CD4^+$ T cells in the head and neck lymph nodes (HNLNs) of ovalbumin (OVA)-immunized mice. The apoptosis of $CD127^{low/-}CD25^+CD4^+$ (A) $CD127^+CD25^+CD4^+$ (B) and $CD25^-CD4^+$ (C) T cells was assessed in the HNLNs of non-immunized mice (PBS), OVA-immunized mice, ciclesonide-treated, OVA-immunized mice (OVA + CIC) and methylprednisolone-treated, OVA-immunized mice (OVA + MP). CIC and MP administration was started 48 h prior to OVA challenge. At the indicated number of days after challenge, mice were sacrificed, and HNLN samples were individually collected. The results represent the percentage of $CD127^{low/-}CD25^+CD4^+$, $CD127^+CD25^+CD4^+$ and $CD25^-CD4^+$ T cells in early apoptosis (Annexin V^+7-AAD^- cells), and they are expressed as the mean (±S.D.) of two independent experiments with 4 mice per group (n = 8 per group, $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).

tract. Moreover, application of inhaled GCs can produce negative effects on lung-residing $CD4^+$ T cells.

Contributions

M.Z.P. and T.M. conceived, designed, and performed the experiments. M.Z.P. analyzed data. M.Z.P. and T.M. interpreted results. M.Z.P. wrote the manuscript. T.M. coordinated the study and helped draft and review the final manuscript. M.Z.P. is the principal author. Both authors read and approved the final manuscript.

Conflict of interest

All authors declare that they have no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.intimp.2017.02.005.

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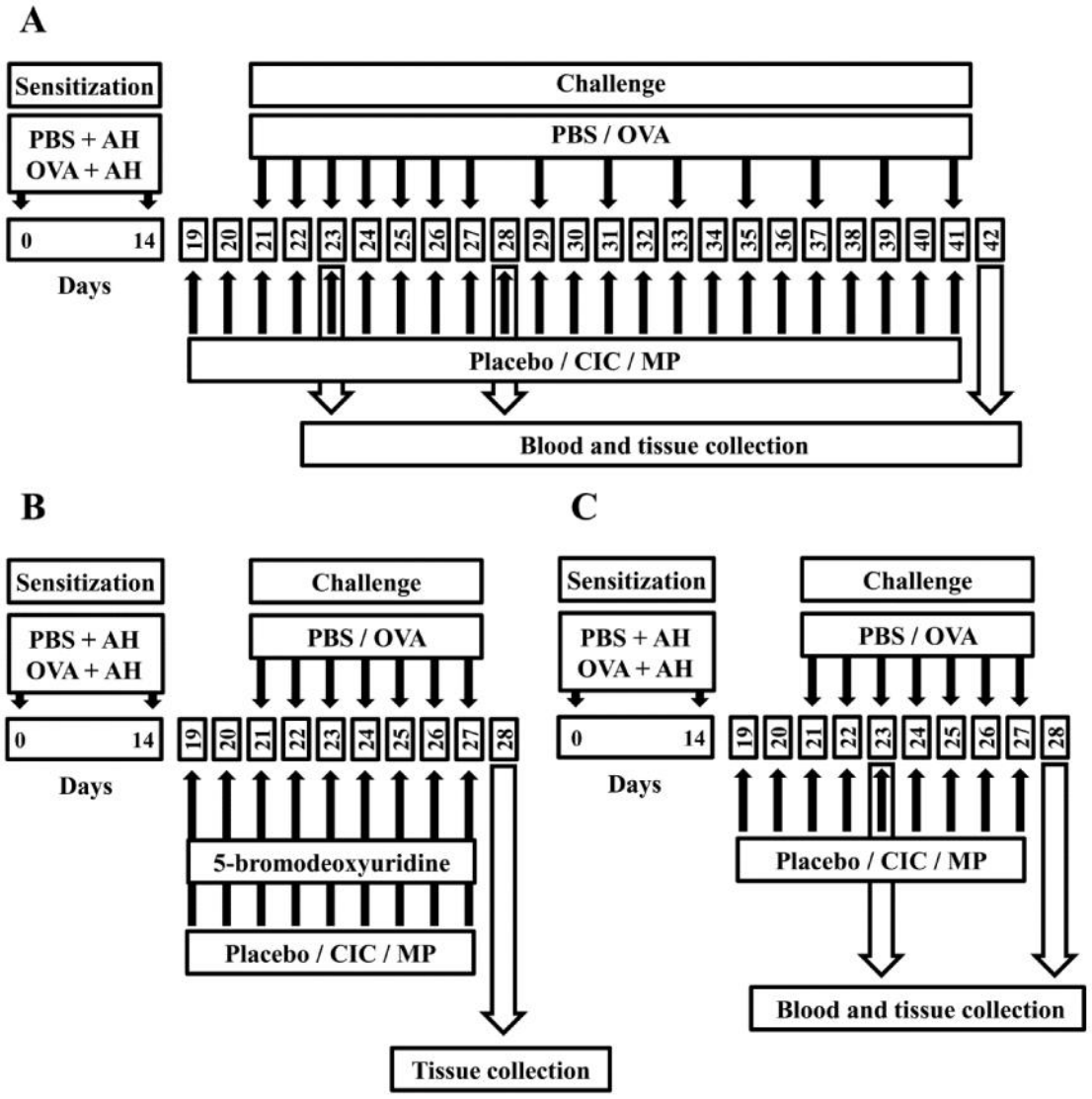
Supplementary materials

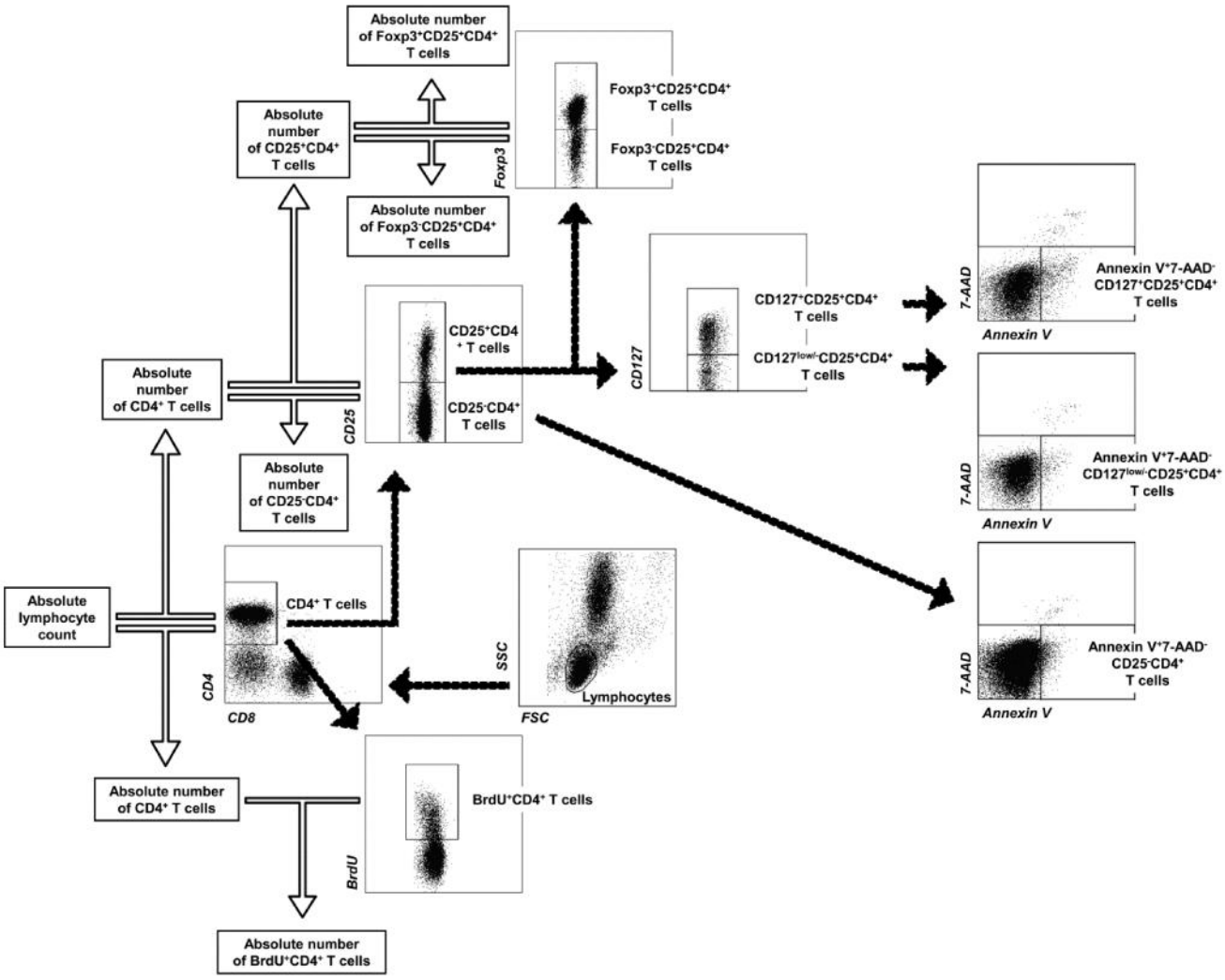
Effect of inhaled and systemic glucocorticoid treatment on CD4$^+$ regulatory and effector T cells in a mouse model of allergic asthma

Monika Zuśka-Prot, Tomasz Maślanka



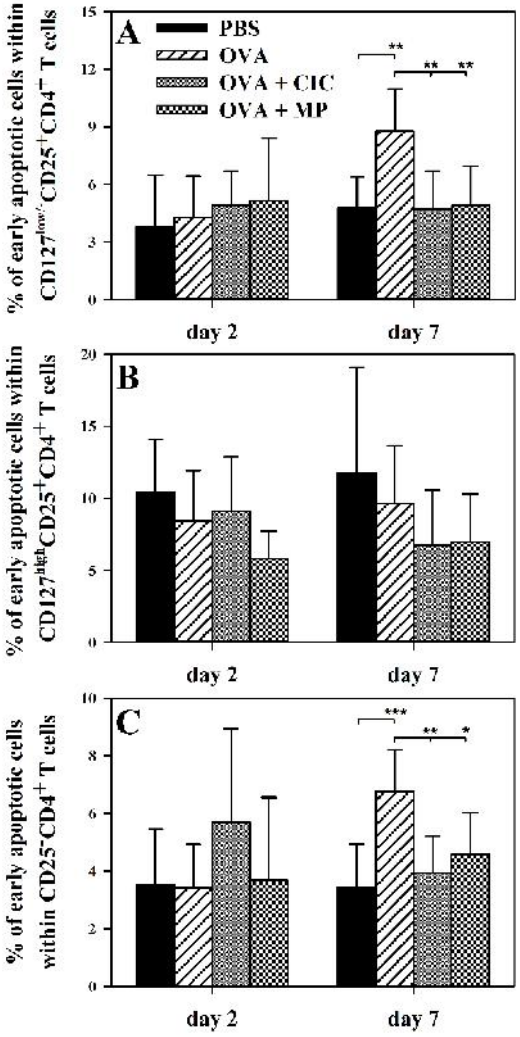
Suplementary figure 1. Experimental study design scheme. The main stages of the study included evaluation of effects of inhaled (Ciclesonide, CIC) and systemic (Methylprednisolone, MP) glucocorticoid therapy on: (a) the absolute counts of Foxp3$^+$CD25$^+$CD4$^+$, Foxp3$^-$CD25$^+$CD4$^+$ and CD25$^-$CD4$^+$ T cells in the mediastinal lymph nodes (MLNs), lungs, head and neck lymph nodes (HNLNs) and peripheral blood (PB); this

parameter was assessed at three time points, i.e. on days 23, 28 and 42 (experiment A); (b) proliferation of $CD4^+$ T cells in the MLNs and lungs; this effect was evaluated at one time point, i.e. on day 28 (experiment B); (c) apoptosis of $CD127^{low/-}CD25^+CD4^+$, $CD127^{high}CD25^+CD4^+$ and $CD25^-CD4^+$ T cells in the MLN, lungs, HNLNs and PB; effect of CIC and MP on apoptosis of studied cells was assessed at two time points, i.e. on days 23 and 28 (experiment C). In each experiment, mice were divided into four groups, namely PBS group (PBS-sensitized and -challenged mice, i.e. negative control), ovalbumin (OVA) group (OVA-sensitized and -challenged mice, i.e. mice with OVA-induced model of allergic asthma/positive control), OVA + CIC group (OVA-sensitized and -challenged mice treated with CIC) and OVA + MP group (OVA-sensitized and -challenged mice treated with MP). Mice were sensitized to OVA by two intraperitoneal (i.p.) injections on days 0 and 14 with OVA absorbed on aluminum hydroxide (AH). Subsequently, mice were challenged intranasally (i.n.) with OVA (OVA, OVA + CIC and OVA + MP groups) once daily on days 21-27; additionally, in experiment A some mice were challenged with OVA every second day from day 27 to 41. Mice in the negative control group (PBS group) received only AH in PBS (sensitization) or PBS alone (challenge). CIC, MP or placebo administration was started 48 h prior to i.n. challenge (i.e. on day 19 after the initial sensitization) and continued for 4 (experiments A and C), 9 (experiments A, B and C) and 23 (experiment A) consecutive days; the drugs or placebo were given once daily 3 h before OVA challenge. For *in vivo* labeling of proliferating cells, mice were injected i.p. with 5-bromo-2′-deoxyuridine (BrdU) every 24 h for 7 days (experiment B). Mice were euthanized 24 h after the last administration of the drugs or placebo, i.e. on day 23 (experiments A and C), 28 (experiments A, B and C) and 42 (experiment A).



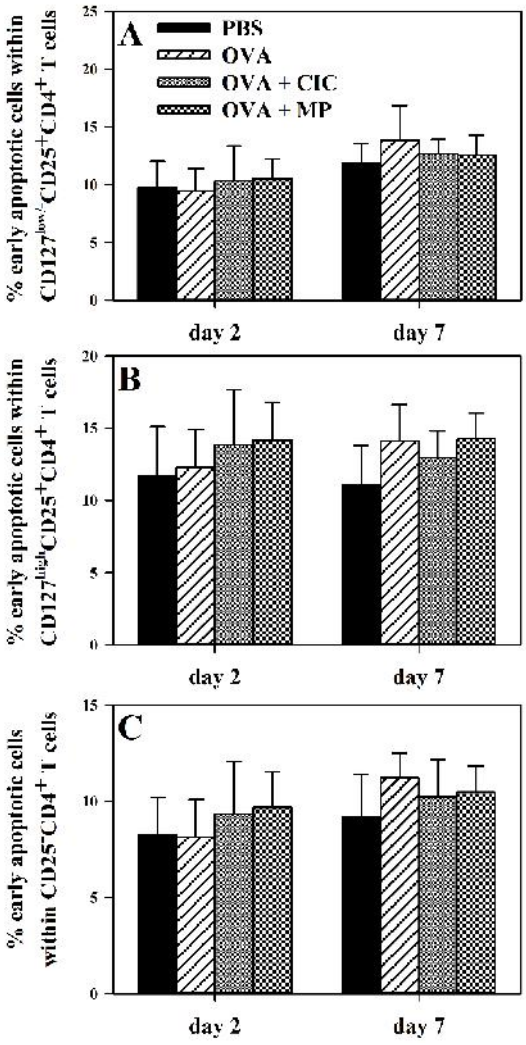
Suplementary figure 2. Gating strategy for FACS analysis and calculation of the absolute cell counts of lymphocyte subsets. Gating strategy for flow cytometric data analysis and calculation of the absolute cell counts of lymphocyte subsets. Lymphocytes were identified based on forward and side scatter (FSC/SSC) properties and then gated for expression of CD4 surface receptors. Relative to the presence of CD25 expression, the $CD4^+$ T cell population was subdivided into $CD25^-$ and $CD25^+$ cell subsets. $CD25^+CD4^+$ T cells were analyzed for Foxp3 and CD127 expression. $CD25^-CD4^+$, $CD127^{low/-}CD25^+CD4^+$ and $CD127^{high}CD25^+CD4^+$ T cell subsets were analyzed for apoptosis by using Annexin V and 7-aminoactinomycin D (7-AAD) staining. Annexin V^-7-AAD^- cells were considered viable; Annexin V^+7-AAD^- cells were deemed early-apoptotic; and double positive cells (Annexin

$V^+7\text{-}AAD^+$) were considered to be a mixture of late apoptotic and primary necrotic cells. Moreover, $CD4^+$ T cells were assessed for proliferation by measuring bromo-2′-deoxyuridine (BrdU) incorporation. Absolute cell counts of lymphocyte subsets (i.e. number of cells from particular subpopulations per µl of blood or per tissue sample) were calculated using the dual platform method, as shown above.



Suplementary figure 3. Effect of inhaled and systemic glucocorticoid therapy on apoptosis of $CD4^+$ T cells in in the mediastinal lymph nodes (MLNs) of ovalbumin (OVA)-immunized mice. The apoptosis of $CD127^{low/-}CD25^+CD4^+$ (A) $CD127^+CD25^+CD4^+$ (B) and $CD25^-CD4^+$ (C) T cells was determined in MLNs of non-immunized mice (PBS), OVA-immunized mice, ciclesonide-treated, OVA-immunized mice (OVA + CIC) and methylprednisolone-treated, OVA-immunized mice (OVA + MP). CIC and MP administration was started 48 h prior to

OVA challenge. At the indicated number of days after challenge, mice were sacrificed, and MLN samples were individually collected. Results represent the percentage of $CD127^{low/-}CD25^{+}CD4^{+}$, $CD127^{+}CD25^{+}CD4^{+}$ and $CD25^{-}CD4^{+}$ T cells in early apoptosis (Annexin V^{+}7-AAD^{-} cells), and they are the mean (± S.D.) of two independent experiments with 4 mice per group (n = 8 per group, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).



Suplementary figure 4. Effect of inhaled and systemic glucocorticoid therapy on apoptosis of $CD4^{+}$ T cells in peripheral blood (PB) of ovalbumin (OVA)-immunized mice. The apoptosis of $CD127^{low/-}CD25^{+}CD4^{+}$ (A) $CD127^{+}CD25^{+}CD4^{+}$ (B) and $CD25^{-}CD4^{+}$ (C) T cells was assessed in PB of non-immunized mice (PBS), OVA-immunized mice, ciclesonide-

treated, OVA-immunized mice (OVA + CIC) and methylprednisolone-treated, OVA-immunized mice (OVA + MP). CIC and MP administration was started 48 h prior to OVA challenge. At the indicated number of days after challenge, mice were sacrificed, and PB samples were individually collected. The results represent the percentage of $CD127^{low/-}CD25^{+}CD4^{+}$, $CD127^{+}CD25^{+}CD4^{+}$ and $CD25^{-}CD4^{+}$ T cells in early apoptosis (Annexin V^{+}7-AAD^{-} cells), and they are expressed as the mean (± S.D.) of two independent experiments with 4 mice per group (n = 8 per group).

A limitation of the studies

Considerable limitation of our experiments lies in the fact that they were conducted on mice. It is known that drugs often exhibit species-dependent properties with regard to pharmacokinetics and pharmacodynamics. Thus, it is obvious that any data obtained from these studies should be extrapolated to humans with great caution.