



Inhaled glucocorticoid treatment prevents the response of CD8⁺ T cells in a mouse model of allergic asthma and causes their depletion outside the respiratory system



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ABSTRACT

The principal objective of this research has been to determine the safety of inhaled glucocorticoids (GCs) in respect of their effect on CD8⁺ T cells within respiratory and extra-respiratory tissues, and to compare it with systemic GC treatment. Another purpose has been to identify whether inhaled and systemic GCs affect the CD8⁺ T cell response in the mediastinal lymph nodes (MLNs) and lungs in a mouse model of ovalbumin (OVA)-induced asthma. Ciclesonide and methylprednisolone were used as a model for inhaled and systemic GCs, respectively. The CD8⁺ T cell response was observed in untreated OVA-immunized mice, manifesting itself by the proliferation of these cells and their recruitment into the lower respiratory tract. Inhaled and systemic GC treatment fully prevented this response. This suggests that one of the elements contributing to the anti-asthmatic efficacy of inhaled and systemic GCs could be the inhibition of the effector CD8⁺ T cell response which accompanies the disease. The anti-asthmatic effect of GCs was rather not mediated through the generation or/and increased recruitment of Foxp3⁺CD25⁺CD8⁺ regulatory T cells into the MLNs and lungs. Inhaled and systemic GCs produced comparable depletions of normal CD8⁺ T cells in the MLNs, the head and neck lymph nodes and in peripheral blood, and this effect, at least to some extent, resulted from the proapoptotic action of GCs towards these cells. These results suggest that inhaled GC therapy might not be safer at all than treatment with systemic GCs in respect of the undesirable effects on CD8⁺ T cells residing within and outside the respiratory tract.

1. Introduction

Inhaled and, in severe cases, systemic glucocorticoids (GCs) are the most effective anti-inflammatory drugs used to control asthma. It is well documented that systemic GCs cause substantial systemic immunosuppression, which is linked to patients becoming more sensitive to various infections. It is widely assumed that inhaled GCs are incomparably safer than systemic GCs with respect to the immunosuppressive action, although there is a lack of broad-scale research focusing on the following question: does inhaled GC therapy produce a negative effect on normal T 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? We have undertaken broader research to elucidate this problem. In our most recent paper, we have described the effects of inhaled and systemic GC treatment on effector and regulatory CD4⁺ T cells in a mouse model of allergic asthma [1]. Ciclesonide (CIC) and methylprednisolone (MP) were used as a model for inhaled and systemic GCs, respectively. Both drug agents are widely used in the treatment of allergic asthma. In that research, we

demonstrated, inter alia, that CIC and MP effectively prevented ovalbumin (OVA)-induced asthma, although the administration of both drugs was associated with the induction of comparable depletion of normal CD4⁺ T cells present within and outside the respiratory tract. In this article, we present results concerning the effect of CIC and MP on effector and regulatory CD8⁺ T cells. These cells are a critical component of the cellular immune response and they play an important role in the immune response against viruses, bacteria and parasites [2]. Thus far, the effect of inhaled GCs on CD8⁺ T cells of peripheral blood in humans concerning the activation of these cells [3–5], their counts [6,7] and their production of IL-5, IL-4 and IFN-γ [5,7] has been investigated. The limitation of these studies lies in the fact that the effect of inhaled GCs on peripheral blood (PB) lymphocytes does not necessarily reflect their effect on T cells in the lower respiratory tissues and associated lymph nodes (LNs), or in the extra-respiratory LNs. The absence of broader comparative studies on the effect of inhaled GCs on CD8⁺ T cells makes a serious gap in knowledge. Therefore, the main aim of this study has been to determine the safety of administration of inhaled GCs in respect of their effect on CD8⁺ T cells within respiratory

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and extra-respiratory tissues, and to compare it with systemic GC treatment.

Although the central role in the pathogenesis of asthma is played by CD4⁺ rather than by CD8⁺ T cells, numerous studies prove that CD8⁺ T cells also participate in the development of the disease. Results of these investigations implicate a dual role of CD8⁺ T cells in the pathogenesis of bronchial asthma. On the one hand, these cells contribute to the exacerbation of asthma [8–12]; on the other hand, the involvement of these cells may contribute to the inhibition of the immune response and inflammation associated with asthma [13,14]. Research with the use of an allergic asthma model has demonstrated the induction of allergen-specific CD8⁺ T cells in response to allergen exposure [15]. In consequence, the number of these cells in the lungs and mediastinal LNs (MLNs) increased [15]. The results reported by Stock et al. [13] and Aguilar-Pimentel et al. [15] strongly suggest that the sources of the increased number of CD8⁺ T cells in the lungs of mice with OVA-induced asthma consisted of both their recruitment into the lungs and proliferation in the tissue itself. Particular attention should be given to reports implicating that the deficiency of Foxp3⁺ CD25⁺ CD8⁺ regulatory T (Treg) cells may also participate in the pathogenesis of asthma [16,17].

The available literature lacks data on the effect of GCs on CD8⁺ T cells involved in the pathogenesis of asthma. Hence, another objective of our investigations was to determine whether inhaled and systemic GCs affected the CD8⁺ T cell response to OVA immunization in the MLNs and lungs, that is in inductive and effector compartments, respectively, associated with allergic asthma. Considering the anti-allergic and immunosuppressive potential of GCs, it was hypothesized that the administration of these drugs would prevent or reduce the above response. Over the recent years, several reports have been published [18–20] suggesting that one of the mechanism responsible for the anti-asthmatic action of GCs could be the generation of Foxp3⁺ CD25⁺ CD4⁺ 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 of Foxp3⁺ CD25⁺ CD8⁺ Treg cells in the lungs and/or in the MLNs.

In the current research, lymphocytes were co-stained for CD8, CD25 and Foxp3 expression so as to identify, and thus distinguish, Treg, activated effector (aTeff) and other cells within the CD8⁺ T cell pool. CD25 (i.e. α chain of the IL-2 receptor) is a marker of recent T cell activation [21], however, it is also expressed on natural and induced Foxp3-expressing CD8⁺ Treg cells [22,23]. Recent research confirms that Foxp3⁺ CD25⁺ CD8⁺ T cells share phenotypic and functional characteristics with CD4⁺ Treg cells and are thus bona fide CD8⁺ Treg cells [24]. Therefore, with a certain measure of simplification, it can be said that cells of the phenotypes Foxp3⁺ CD25⁺ CD8⁺ and Foxp3[−] CD25⁺ CD8⁺ represent Treg and aTeff cells, respectively. In turn, the CD25[−] CD8⁺ T cell subset should be considered primarily as a pool of resting T (Trest) cells, i.e. effector memory, central memory and naïve T cells.

2. Materials and methods

2.1. Animals and Ethics statement

Experiments were performed on 6-week-old female BALB/c mice. The animals were housed and treated in accordance with the rules of the Local Ethics Commission for Animal Experiments in Olsztyn, as described previously [1]. All of the procedures were approved by the Local Ethics Commission (Ethic permission No. 35/2014/N). Mice were euthanized by asphyxiation with CO₂.

2.2. Antigen immunization, airway challenge and treatment protocol

Mice were randomly divided into four groups: PBS (phosphate buffered saline) group (PBS-sensitized and -challenged mice), OVA

group (OVA-sensitized and -challenged mice), OVA + CIC group (OVA-sensitized and -challenged mice treated with CIC) and OVA + MP group (OVA-sensitized and -challenged mice treated with MP). As described in detail previously [1], mice were sensitized on days 0 and 14 via intraperitoneal (i.p.) injection of 20 μ g OVA emulsified in 2 mg aluminum hydroxide (AH) (both from Sigma-Aldrich, Schnellendorf, Germany). Mice were challenged intranasally (i.n.), once a day, with 100 μ g of OVA in PBS. Mice in the PBS group received only AH in PBS (sensitization) or PBS alone (challenge). A schematic diagram of the study design is shown in Supplementary Fig. S1.

As described previously [1], individual mice from OVA + CIC group were placed into an inhalation chamber, where they were exposed daily to a single dose of 160 μ g (i.e. one puff) inhaled CIC (Alvesco, Takeda GmbH, Konstanz, Germany) for 15 min. Mice from the remaining groups were placed in the inhalation chamber for the same period of time, but were not exposed to CIC. MP (Solu-Medrol, Pfizer Manufacturing Belgium NV, Puurs, Belgium) was dissolved in saline solution and injected intramuscularly (i.m.) at a dose 2 mg/kg/day, i.e. the dose which is used in the maintenance treatment of asthma. The animals from PBS, OVA and OVA + CIC groups were treated i.m. with 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 type of the experiment; the drugs 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. Mice were euthanized at the appropriate time points, as it is shown in Supplementary Fig. S1. Furthermore, additional experiments were carried to investigate the effect of CIC and MP on CD8⁺ T cells in non-immunized mice. To this aim, mice were divided into 3 groups: control (CON) group (non-treated mice), CIC group (mice treated with ciclesonide) and MP group (mice treated with methylprednisolone). The drugs were administered every 24 h for 9 days (which corresponds to the duration of the administration of drugs to OVA-immunized mice as of day 28 of the experiment) 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).

2.3. Cell recovery

2.3.1. MLNs and HNLNs

MLN and HNLN lymphocytes were isolated as previously described [1,25]. Whole set of MLNs and HNLNs were removed and subjected to homogenization with Dounce tissue grinder. 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 Ca²⁺ and Mg²⁺ with 2% (v/v) heat-inactivated FBS (both from Sigma-Aldrich)], and centrifuged (300 $\times$ 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.

2.3.2. Lung samples

Because the cell yield from lung samples of a single mouse was 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 [25]. With respect to the evaluation of apoptosis, the collagenase step was omitted because the digestion process could cause false positive results.

2.3.3. PB

Blood was drawn from the inferior vena cava into heparinized or and EDTA coated tubes. Erythrocytes were removed as previously

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

Staining combinations	Clone	Isotype	Evaluated parameters
APC-Cy7/anti-mouse CD8a	53–6.7	IgG2a, κ	The absolute number of: • Foxp3 ⁺ CD25 ⁺ CD8 ⁺ T cells • Foxp3 [−] CD25 ⁺ CD8 ⁺ T cells • CD25 [−] CD8 ⁺ T cells
PE-Cy7/anti-mouse CD25	PC61	IgG1, λ	
PE/anti-mouse Foxp3	MF23	IgG2b	
APC-Cy7/anti-mouse CD8a	53–6.7	IgG2a, κ	
PE/Annexin V	-	-	The percentage of early apoptotic (Annexin V ⁺ 7-AAD [−]) cells within CD8 ⁺ T cell subset
7-AAD	-	-	
APC-Cy7/anti-mouse CD8a	53–6.7	IgG2a, κ	The absolute count of proliferating (BrdU ⁺) CD8 ⁺ T cells
APC/anti-BrdU	BU1/75	IgG2a	

described [25]. The remaining cells were re-suspended in FB and stained for flow cytometric analysis. Routine complete blood count (CBC) was performed using a hematology analyzer Advia 2120i (Siemens Healthcare Diagnostics, Erlangen, Germany).

2.4. Staining for flow cytometry analysis

2.4.1. Extracellular staining

Cells resuspended in FB were labeled for surface antigens with fluorochrome conjugated monoclonal antibodies (mAbs) specific to mouse CD8 and CD25 (both from BD Biosciences, San Jose, CA, USA), as demonstrated in Table 1. After 45 min incubation, the cells were washed with 2 ml of FB. The staining combinations, properties of antibodies used in the experiments and evaluated parameters are summarized in Table 1.

2.4.2. 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 [25].

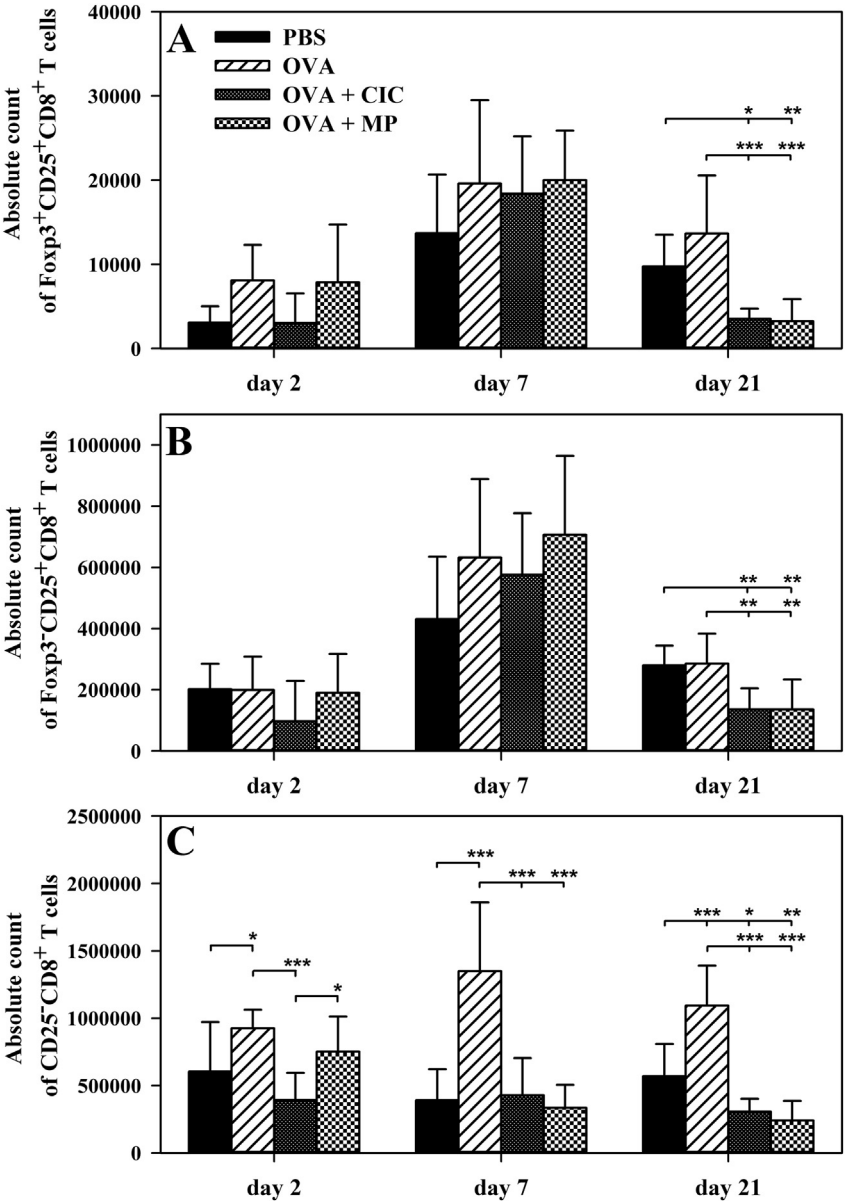


Fig. 1. Effect of inhaled and systemic glucocorticoid treatment on the number of CD8⁺ T cells in the mediastinal lymph nodes (MLNs) of ovalbumin (OVA)-immunized mice. The absolute counts of Foxp3⁺ CD25⁺ CD8⁺ (A) Foxp3[−] CD25⁺ CD8⁺ (B) and CD25[−] CD8⁺ (C) T cells were determined in the MLNs of untreated, non-immunized mice (PBS), untreated, OVA-immunized mice (OVA), ciclosonide-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).

2.4.3. Staining for the evaluation of apoptosis

Cells were stained for CD8 were labeled for apoptosis detection with annexin V and 7-AAD as previously described [26].

2.4.4. Staining for the evaluation of proliferation

Cells stained for CD8 were labeled for incorporated BrdU according to the manufacturer's procedure (APC BrdU Flow Kit, BD Biosciences).

2.5. FACS acquisition and analysis

Flow cytometric analysis was performed as previously described [1,25]. Briefly, samples were acquired on the FACSCanto II cytometer (BD Biosciences), and data was analyzed using the FlowJo software (Tree Star Inc., Stanford, USA). Absolute cell counts of lymphocyte subsets 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.

2.6. Statistical analysis

Data were expressed as the mean ($\pm$ 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 . SigmaPlot Software Version 12.0 (Systat Software Inc., San Jose, USA) was used for statistical analysis and the plotting of graphs.

3. Results

3.1. In the MLNs, CIC and MP prevent OVA-induced increase in the absolute count of CD25⁺CD8⁺ T cell subset

No significant changes occurred in counts of Foxp3⁺CD25⁺CD8⁺ and Foxp3⁺CD25⁺CD8⁺ T cells in the MLNs of mice from the OVA group (Fig. 1A and B). In turn, at all time points, the absolute count of CD25⁺CD8⁺ T cells in untreated OVA-immunized mice was significantly higher than the values of this parameter achieved in non-immunized animals, whereas the administration of CIC and MP prevented this effect (Fig. 1C). Long-term exposure to CIC and MP led to depletion of all studied CD8⁺ T cell subsets in the MLNs (Fig. 1A–C).

3.2. CIC and MP prevents an OVA-induced increase in the absolute count of all studied CD8⁺ T cell subsets in the lungs

The absolute number of all studied subsets was considerably higher in the lungs of untreated OVA-immunized mice, and this effect was rapid and lasting in character, as it appeared at both the second and third time point (Fig. 2A–C). The administration of CIC and MP prevented this effect, because the absolute counts of Foxp3⁺CD25⁺CD8⁺, Foxp3⁺CD25⁺CD8⁺ and CD25⁺CD8⁺ T cells did not differ from the counts of these cells in healthy animals, while the values of these parameters were significantly lower than in untreated OVA-immunized mice (Fig. 2A–C).

3.3. Administration of OVA and co-administration of OVA and CIC or MP lead to a depletion of CD25⁺CD8⁺ T cells in the HNLNs

At the final time point, the absolute counts of Foxp3⁺CD25⁺CD8⁺ and CD25⁺CD8⁺ T cells in untreated OVA-immunized mice were significantly lower than the values obtained in non-immunized animals (Fig. 3A and C). On day 7 and 21 of the experiment, the absolute count of CD25⁺CD8⁺ T cells in the HNLNs of CIC- and MP-treated OVA-immunized mice was significantly reduced (Fig. 3C). Moreover, a

significant reduction in the absolute count of Foxp3⁺CD25⁺CD8⁺ T cells was noted at 3rd time point in CIC-treated mice. Treatment with MP prevented an OVA-induced reduction in Foxp3⁺CD25⁺CD8⁺ T cells counts in HNLNs (Fig. 3A).

3.4. Both immunization with OVA and co-administration of OVA and CIC or MP induce depletion of CD8⁺ T cells in PB

At the second time point, the absolute counts of all studied subsets in untreated OVA-immunized mice were significantly lower than the values obtained in non-immunized animals (Fig. A–C). With respect to Foxp3⁺CD25⁺CD8⁺ T cells, this change was a lasting one because it persisted at the third time point (Fig. 4C). It was found that the absolute counts of almost all studied subsets were significantly lower in the CIC- and MP-treated OVA-immunized mice at 2nd and 3rd time point compared to the values obtained in non-immunized animals.

3.5. Administration of CIC to non-immunized mice leads to depletion of CD8⁺ T cells in the MLNs, HNLNs and PB

The studies revealed a depletion of CD8⁺ T cells outside the respiratory system in both untreated OVA-immunized mice and in the CIC- or MP-treated OVA-immunized mice. Therefore, in order to distinguish the effects of immunization from the influence of the drugs, it was necessary to conduct studies consisting in the administration of CIC and MP to non-immunized mice. CIC did not affect the absolute count of Foxp3⁺CD25⁺CD8⁺ T cells in the lungs, HNLNs and PB, although it caused a significant reduction of these cells in the MLNs (Fig. 5A–D). Moreover, CIC induced a depletion of Foxp3⁺CD25⁺CD8⁺ T cells in the MLNs and PB, but not in the lungs and HNLNs (Fig. 5A'–D'). The strongest effect of the inhaled GC treatment was produced on CD25⁺CD8⁺ T cells, because treatment with CIC induced a loss of these cells in all the analyzed tissues (Fig. 5A''–D''). In turn, the administration of MP to non-immunized mice had no effect on the absolute number of Foxp3⁺CD25⁺CD8⁺ T cells in the MLNs, lungs and PB, but induced their depletion in the HNLNs (Fig. 5A–D). Systemic GC treatment caused a reduction of the absolute count of Foxp3⁺CD25⁺CD8⁺ T cells in both analyzed LNs, but not in the lungs or PB (Fig. 5A'–D'). The strongest effect of MP was produced on CD25⁺CD8⁺ T cells because the administration of the drug significantly reduced the number of these cells in the MLNs, HNLNs and PB (Figs. 5A'', C'' and D''). Contrary to CIC, the exposure to MP did not cause a depletion of CD25⁺CD8⁺ T cells in the lungs (Fig. 5B'').

3.6. Administration of CIC or MP prevents OVA-induced proliferation of CD8⁺ T cells in the MLNs

The study revealed that OVA immunization enhanced the proliferation of CD8⁺ T cells in the MLNs because the absolute count of BrdU⁺CD8⁺ T cells in this tissue, and additionally in the lungs, was significantly higher than determined in non-immunized mice (Fig. 6). Administration of CIC or MP prevented this effect as the number of BrdU⁺CD8⁺ T cells in the MLNs (Fig. 6A) and lungs (Fig. 6B) of GC-treated mice was significantly lower than in the OVA-group, and did not differ significantly from the value of this parameter achieved in non-immunized mice.

3.7. Exposure to CIC induces an increase in the percentage of early apoptotic cells within CD8⁺ T cells in the lungs and HNLNs

In the MLNs of untreated OVA-immunized mice, the percentage of early apoptotic cells within CD8⁺ T cells was significantly higher than in non-immunized and GC-treated mice (Fig. 7A). Treatment with CIC caused a significant increase in the percentage of early apoptotic cells within CD8⁺ T cells in the lungs and HNLNs (Fig. 7B and C). An increase in this parameter was also observed in the HNLNs of mice

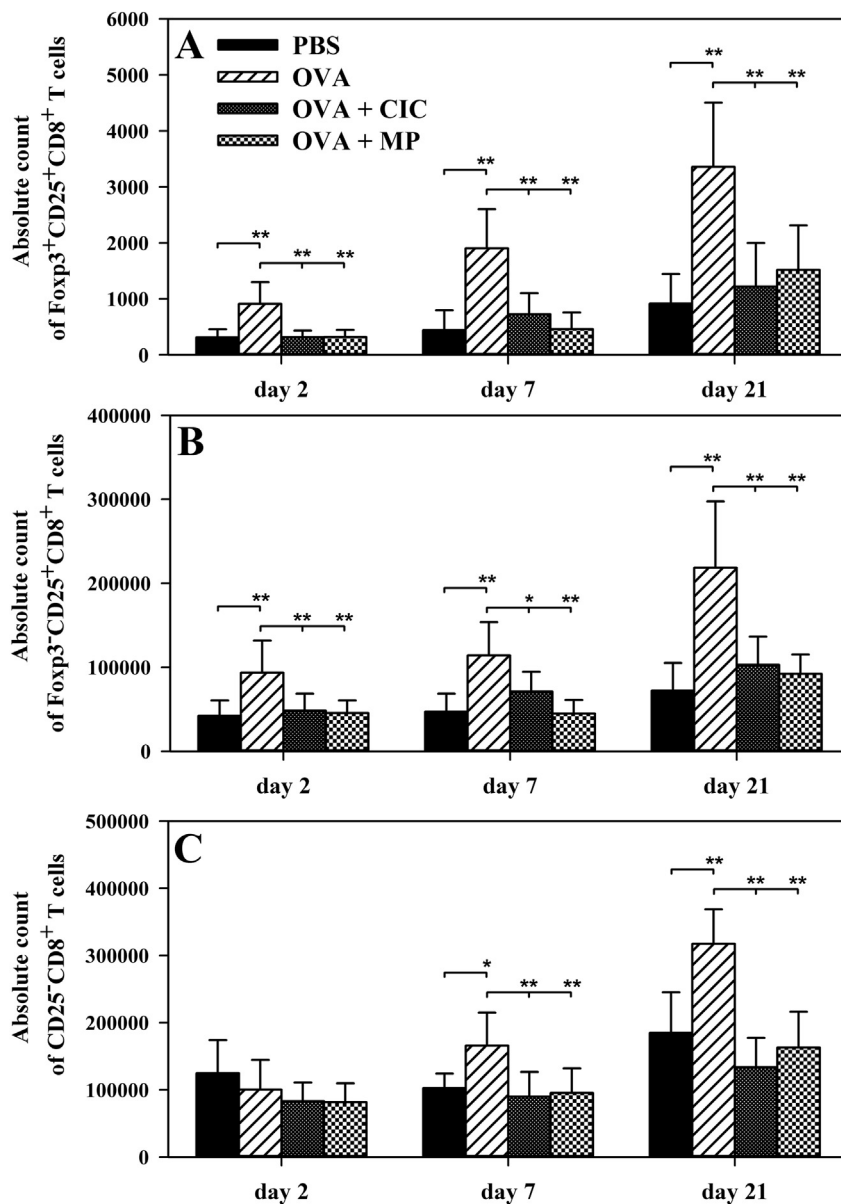


Fig. 2. Effect of inhaled and systemic glucocorticoid treatment on the number of CD8⁺ T cells in the lungs of ovalbumin (OVA)-immunized mice. The absolute counts of Foxp3⁺CD25⁺CD8⁺ (A), Foxp3⁻CD25⁺CD8⁺ (B) and CD25⁻CD8⁺ (C) T cells were assessed in the lungs of untreated, non-immunized mice (PBS), untreated, 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 ($\pm$ S.D.) of two independent experiments involving 5 lung samples per group ($n = 10$ per group, $*P < 0.01$, $**P < 0.001$).

administered MP (Fig. 7C). CIC or MP was not determined to have an influence on the percentage of early apoptotic cells within PB CD8⁺ T cells (Fig. 7D).

4. Discussion

4.1. CD8⁺ T cell response to OVA in the MLNs and lungs

Immunization of untreated mice with OVA caused a significant increase in the number of CD25⁻CD8⁺ T cells in the MLNs, but had no effect on the counts of CD8⁺ Treg or aTeff cells in this tissue. The results suggest that the said increase could have been caused not only by the proliferation of CD8⁺ T cells in the MLNs, but also an elevated recruitment of these cells into the MLNs. This is suggested by the fact that the number of CD8⁺ T cells in the MLNs of untreated OVA-immunized mice was about 3.5-fold higher than the control values (day 7), while the number of BrdU⁺CD8⁺ T cells was just twice as high in these animals than in the non-immunized mice. The results obtained by Stock et al. [13] also indicate that there was infiltration of CD8⁺ T cells into the peribronchial LNs in an OVA-induced mouse asthma model. These researchers, as well as others [15], also noted an increase in the

number of CD8⁺ T cells in the lungs of mice with OVA-induced asthma. These findings are in accord with our results, where we demonstrated a permanent rise in the number of CD8⁺ Treg, aTeff and Trest cells in the lungs of untreated OVA-immunized mice. This effect most clearly was a consequence of the recruitment of these cells into the lungs and, possibly, local proliferation of CD8⁺ Teff cells. The latter possibility seems to be suggested by the elevated number of BrdU⁺CD8⁺ T cells in the lungs of untreated OVA-immunized mice, and additionally is supported by results of other research [15]. However, it cannot be ruled out that CD8⁺ T cells could have incorporated BrdU outside the lungs and only afterwards infiltrate this organ. To the best of our knowledge, the current study is the first one to reveal that Foxp3⁺CD25⁺CD8⁺ Treg cells are upregulated in the lungs in a mouse model of OVA-induced asthma.

4.2. Effects of inhaled and systemic GCs on CD8⁺ T cell response to OVA

Administration of either CIC or MP prevented an OVA-induced increase in the number of CD8⁺ T cells in the MLNs and lungs, as well as OVA-induced proliferation of CD8⁺ T cells. No differences were detected in the strength of the effect produced by CIC and MP in this

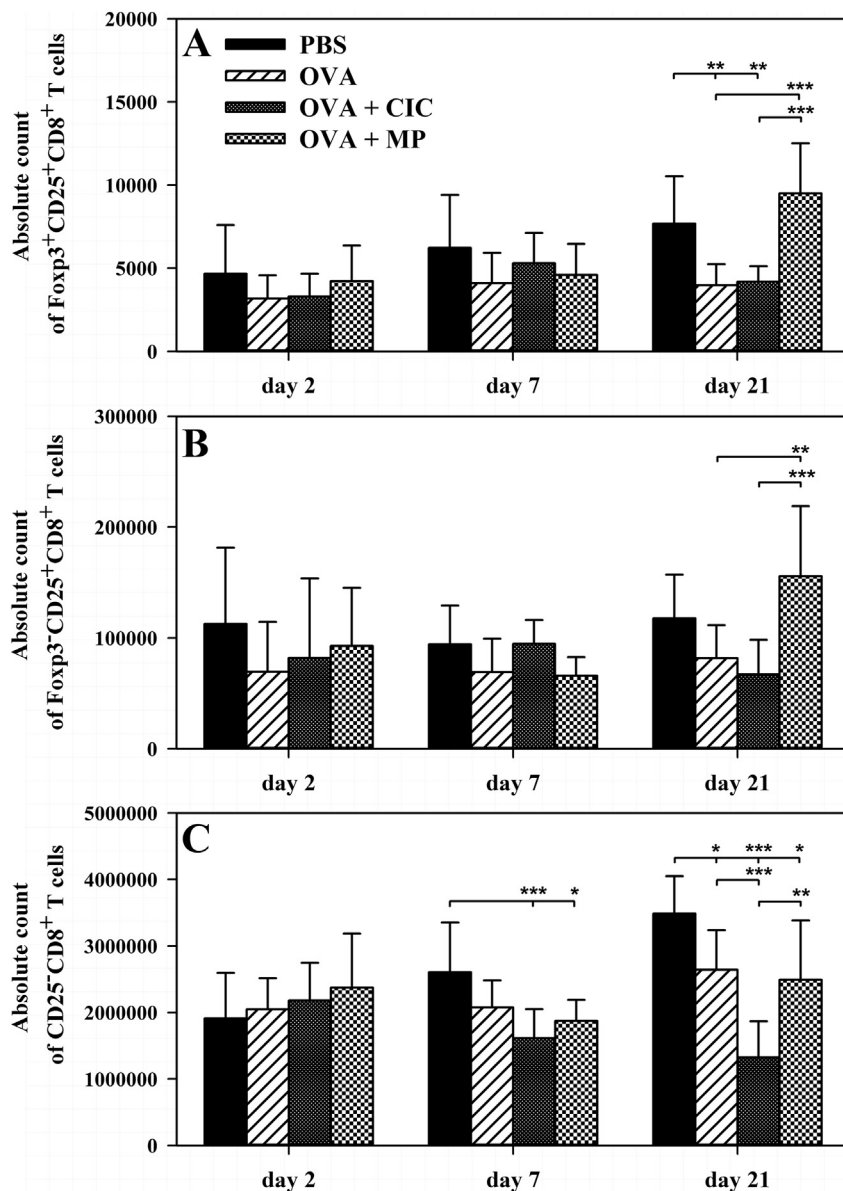


Fig. 3. Effect of inhaled and systemic glucocorticoid treatment on the number of CD8⁺ T cells in the head and neck lymph nodes (HNLNs) of ovalbumin (OVA)-immunized mice. The absolute counts of Foxp3⁺CD25⁺CD8⁺ (A) Foxp3⁻CD25⁺CD8⁺ (B) and CD25⁻CD8⁺ (C) T cells were determined in the HNLNs of untreated, non-immunized mice (PBS), untreated, 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 ($\pm$ S.D.) of two independent experiments with 5 mice per group ($n = 10$ per group, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$).

respect. Hence, the results seem to suggest that the administration of both inhaled and systemic GCs fully prevented the recruitment of CD8⁺ T cells into the lower respiratory tract and their proliferative response to the allergen. Because of the dual role of CD8⁺ T cells in the pathogenesis of allergic asthma, it is impossible to provide unambiguous interpretation of the results in the clinical context. However, in light of numerous reports indicating the involvement of CD8⁺ T cells in the exacerbation of allergic asthma [8–12], an assumption can be made that the inhibition of the effector CD8⁺ T cell response that accompanies the disease is one of the many elements constituting the anti-asthmatic efficacy of inhaled and systemic GCs. The results obtained in this study suggest that the anti-asthmatic effect of these agents was not associated with the generation/recruitment of Foxp3-expressing CD8⁺ Treg cells in the lungs and/or in the MLNs.

4.3. Effects of inhaled and systemic GCs on CD8⁺ T cells in the context of undesirable effects

Long-, but not short-term, administration of CIC and MP to OVA-immunized mice caused depletion of all analyzed CD8⁺ T cells in the MLNs. Same effects occurred much earlier when the drugs were given

to non-immunized animals. The cyto-reductive effect was observed in the lungs of non-immunized mice treated with CIC, but not with MP, and involved only CD8⁺ Trest cells. Interestingly, no such effect was detected in OVA-immunized animals. A possible reason for such varied responsiveness of lung-residing CD8⁺ T cells to CIC between immunized and non-immunized mice might have arisen from the larger exposure of the latter group of animals to the drug. Several studies have demonstrated that the resulting systemic exposure after treatment with some inhaled GCs is lower in asthma patients when compared with healthy subjects [27]. The present studies showed that not only systemic but also inhaled GC treatment induced a loss of CD8⁺ T cells outside the respiratory system. Moreover, inhaled but not systemic GC caused a larger depletion of CD8⁺ Trest cells in the HNLNs of OVA-immunized mice. The said effect was not caused by immunization but by the administration of GCs because (1) this effect also appeared in non-immunized mice, (2) at the second time point, this effect occurred in CIC- and MP-treated OVA-immunized mice, but not in the untreated animals, and (3) the depletion of CD8⁺ Trest cells was significantly larger in animals exposed to the effect of CIC for a long time compared to untreated OVA-immunized mice. The administration of either of the GCs tested caused a permanent depletion of CD8⁺ aTeff and Trest cells

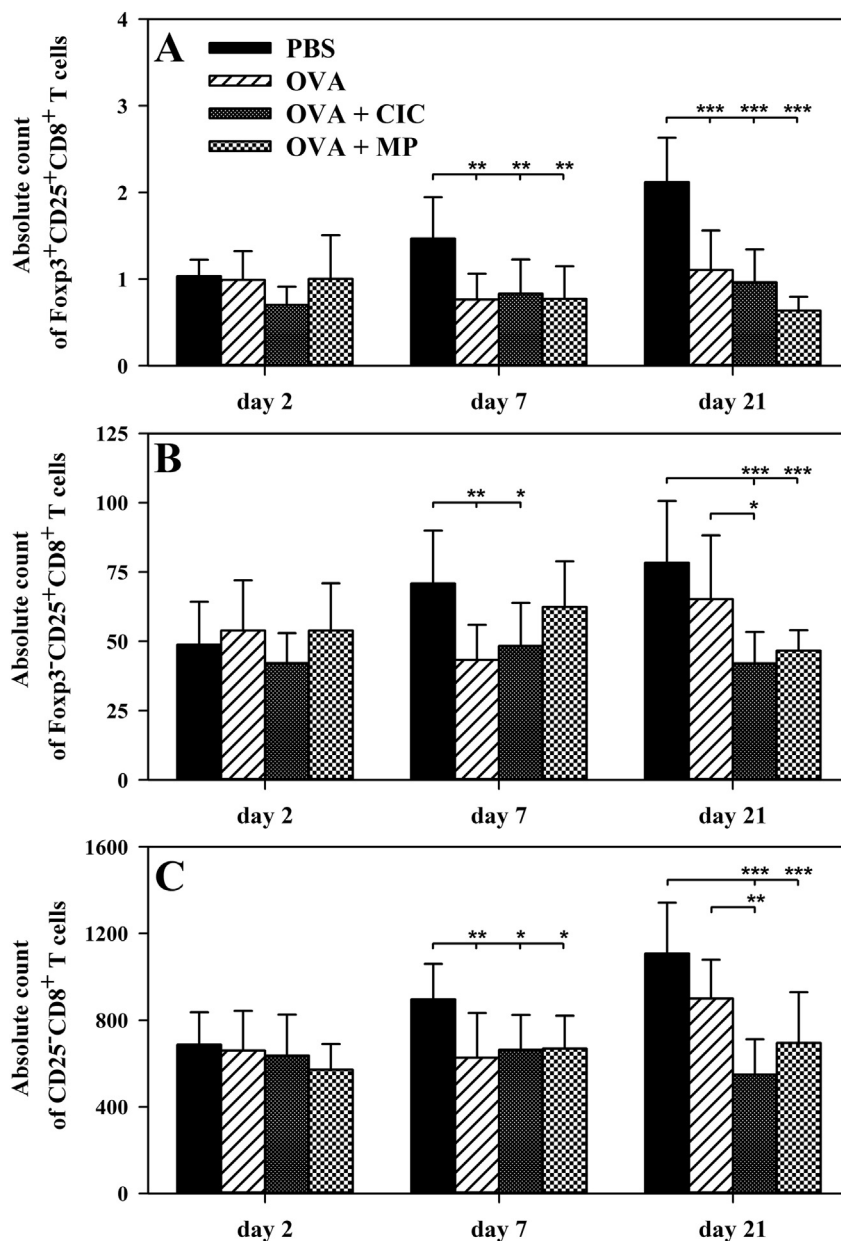


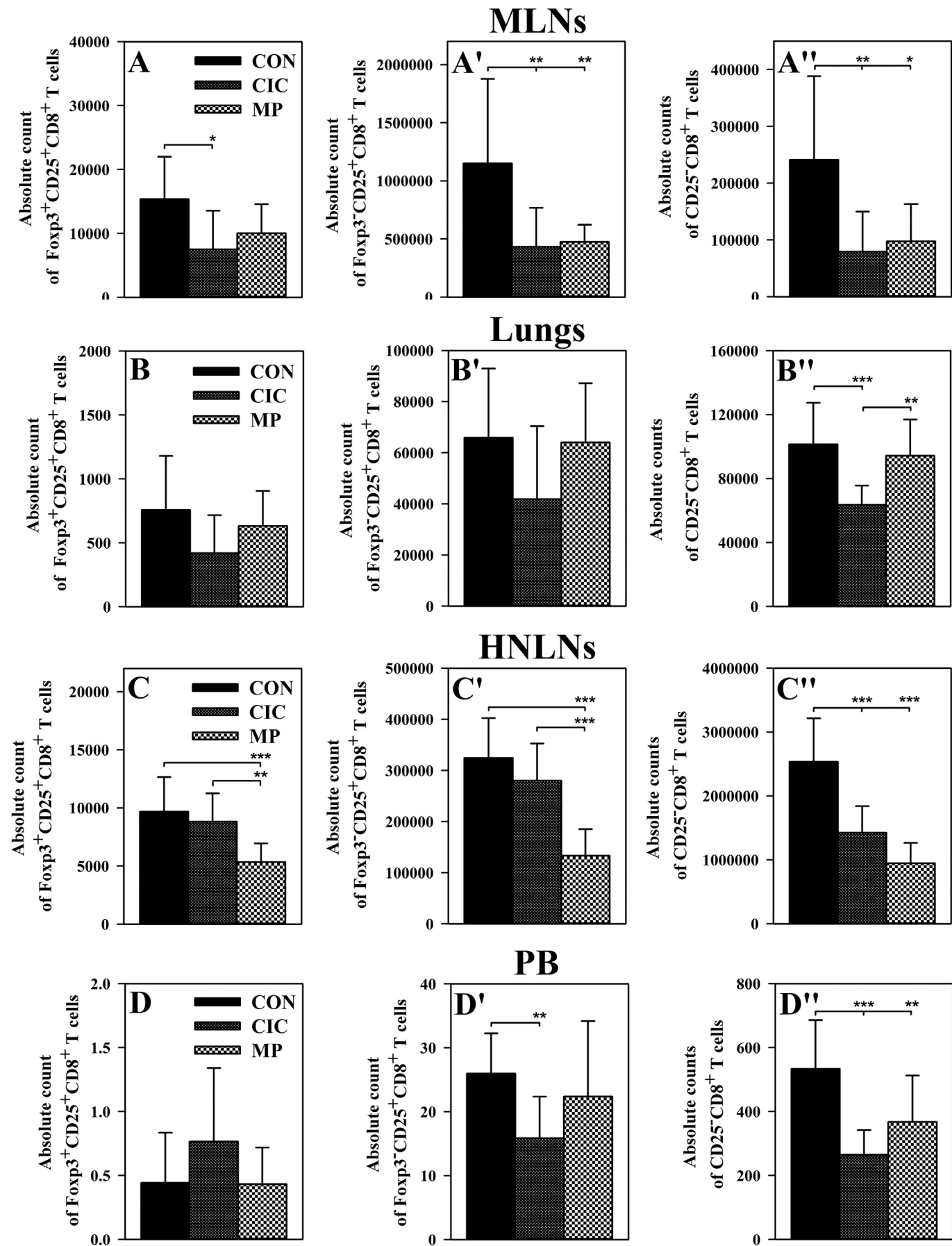
Fig. 4. Effect of inhaled and systemic glucocorticoid treatment on the number of CD8⁺ T cells in peripheral blood (PB) of ovalbumin (OVA)-immunized mice. The absolute counts of Foxp3⁺ CD25⁺ CD8⁺ (A), Foxp3⁻ CD25⁺ CD8⁺ (B) and CD25⁻ CD8⁺ (C) T cells were assessed in PB of untreated, non-immunized mice (PBS), untreated, 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 μ l of blood. Results are expressed as the mean ($\pm$ S.D.) of two independent experiments with 5 mice per group ($n = 10$ per group, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

in PB in the OVA-immunized mice. Interestingly, CIC rather than MP more rapidly induced the loss of CD8⁺ aTeff cells. Most clearly, this effect was not caused by immunization because (1) at the final time point the depletion of these cells was not observed in untreated OVA-immunized mice, while it persisted in animals treated with the drugs, and (2) administration of CIC and MP in non-immunized mice led to a depletion of CD8⁺ Trest and aTeff (only CIC) cells. Unfortunately, the results do not allow us to conclude whether the depletion of CD8⁺ Treg cells in the HNLNs and PB in OVA-immunized mice was caused by immunization or the administration of CIC (HNLNs and PB) and MP (PB).

The available literature contains very few reports [6,7] on the effect of GCs on the absolute number of CD8⁺ T cells. Kawayama et al. [7], having administered CIC in doses of 40 and 80 μ g to asthma patients for seven days, did not demonstrate any influence of this drug on the above parameter in PB. This discrepancy between the results reported by Kawayama et al. [7] and ours could arise from differences in the drug doses tested in the experiments. The cytoreductive effect of inhaled GCs can be dose-dependent, as it has been demonstrated that budesonide in

a high dose, but not a low one, caused depletion of CD8⁺ T cells in PB [6]. The results indicate that neither inhaled nor systemic GCs generate Foxp3⁺ CD25⁺ CD8⁺ Treg cells, as was previously postulated with regard to Foxp3⁺ CD25⁺ CD4⁺ Treg cells [18–20]. Furthermore, the research has also implicated that GCs can cause depletion of these cells.

A question arises as to the sources/mechanisms behind the OVA- and GC-induced depletion of CD8⁺ T cells within and outside the respiratory system. The depletion of CD8⁺ T cells in the HNLNs and PB of untreated OVA-immunized animals was most probably a consequence of their recruitment into the respiratory tissue and LNs. In turn, the loss of normal CD8⁺ T cells within and outside the respiratory system in the CIC- and MP-treated OVA-immunized mice was, at least to some extent, a result of the proapoptotic effect of these drugs. The administration of either of the two GCs tested induced the apoptosis of CD8⁺ T cells in the HNLNs. However, we were unable to demonstrate the proapoptotic effect of CIC and MP towards CD8⁺ T cells in the MLNs and PB, but it is very difficult to capture this effect under *in vivo* conditions. The application of CIC, but not of MP, induced the apoptosis of CD8⁺ T cells in the lungs, which is consistent with the fact that it was the inhaled but



(caption on next page)

Fig. 5. Effect of inhaled and systemic glucocorticoid treatment on the number of CD8⁺ T cells in the mediastinal lymph nodes (MLNs), lungs, head and neck lymph nodes (HNLNs) and peripheral blood (PB) of non-immunized mice. The absolute counts of Foxp3⁺CD25⁺CD8⁺ (A, B, C and D), Foxp3[−]CD25⁺CD8⁺ (A', B', C' and D') and CD25[−]CD8⁺ (A'', B'', C'' and D'') T cells were assessed in the MLNs (A, A' and A''), lungs (B, B' and B''), HNLNs (C, C' and C'') and PB (D, D' and D'') of non-treated mice (CON) and ciclesonide (CIC) or methylprednisolone-treated mice (MP). CIC and MP were administered once daily for 9 days (which corresponds to the duration of the administration of drugs to OVA-immunized mice as of day 28 of the experiment), 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).

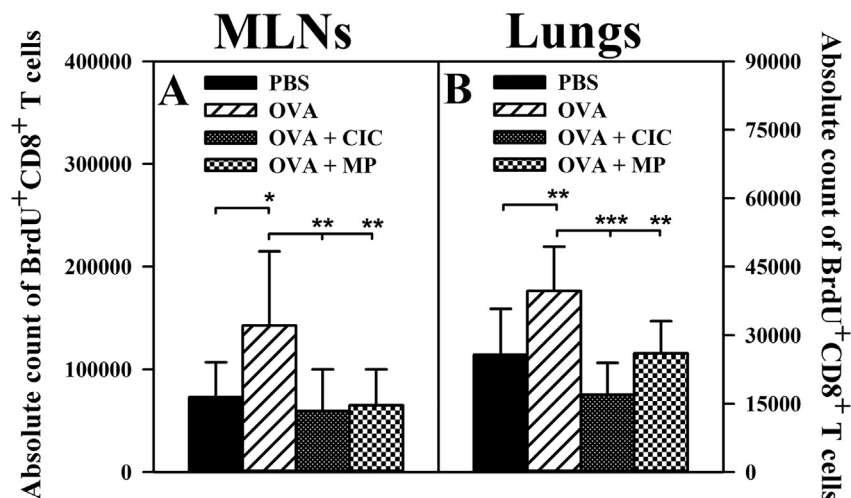


Fig. 6. Effect of inhaled and systemic glucocorticoid treatment on the number of proliferating CD8⁺ T cells in the mediastinal lymph nodes (MLNs) (A) and lungs (B) of ovalbumin (OVA)-immunized mice. The absolute counts of proliferating (BrdU⁺) CD8⁺ T cells were determined in the MLNs (A) and lungs (B) of untreated, non-immunized mice (PBS), untreated, 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⁺CD8⁺ 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 < 0.05, **P < 0.01, ***P < 0.001).

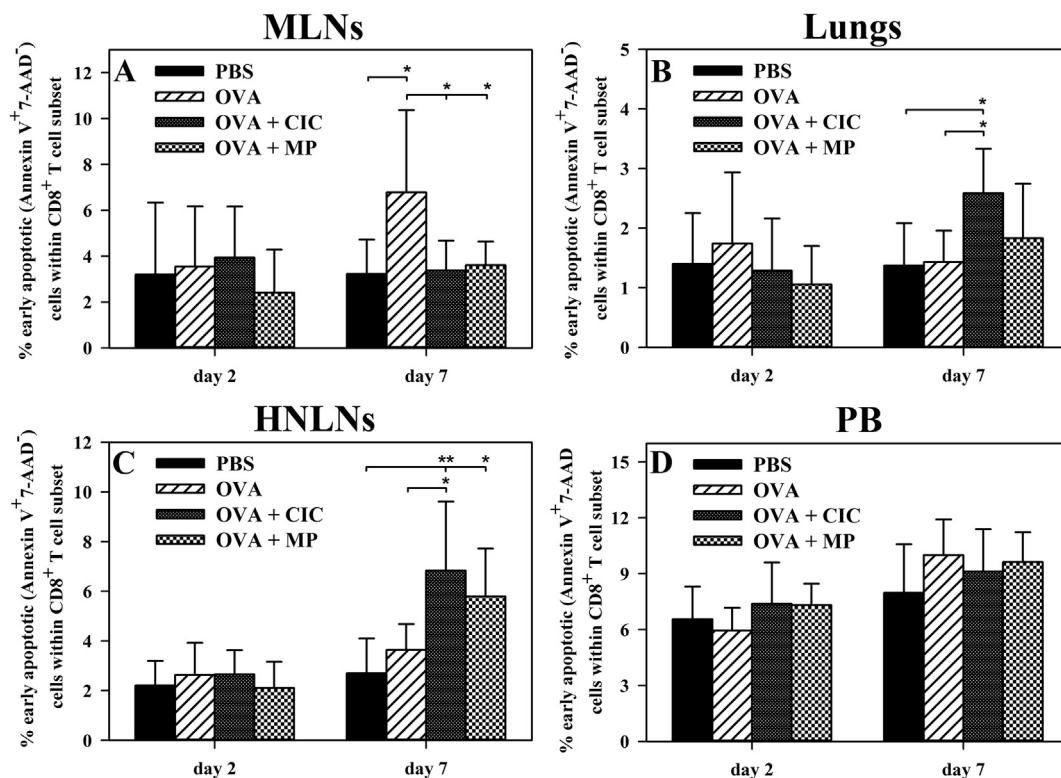


Fig. 7. Effect of inhaled and systemic glucocorticoid treatment on apoptosis of CD8⁺ T cells in the mediastinal lymph nodes (MLNs) (A), lungs (B), head and neck lymph nodes (HNLNs) (C) and peripheral blood (PB) (D) of OVA-immunized mice. The apoptosis of CD8⁺ T cells was determined in the MLNs, lungs, HNLNs and PB of untreated, non-immunized mice (PBS), untreated, 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 MLN samples were individually collected. Results are expressed as a percentage of early apoptotic cells (Annexin V⁺7-AAD[−] cells) within CD8⁺ T cell subset. Results are the mean (± S.D.) of two independent experiments involving 4 lung samples (each lung sample consisted of cells pooled from three mice) or 4 mice (MLNs, HNLNs and PB) per group (n = 8 per group, *P < 0.05, **P < 0.001).

not the systemic GC treatment that induced depletion of these cells in the lungs of non-immunized mice. Considering the fact that both GCs prevented OVA-induced recruitment/proliferation of CD8⁺ T cells in lungs, it seems justified to suggest that the proapoptotic effect of

inhaled GCs therapy most likely involved normal lung-residing CD8⁺ T cells but not the lung infiltrating, pro-inflammatory CD8⁺ T cells.

The research results suggest that inhaled GCs may cause depletion of CD8⁺ T cells residing within respiratory and extra-respiratory

tissues, but the intensity of this disorder can be comparable to the one that occurs during the maintenance treatment with systemic GCs. CD8⁺ T cells are a crucial component of the cellular immune response, which is necessary for the control of a variety of bacterial and viral infections. Hence, inhaled GCs can have a negative effect on T cells involved in the production of cellular immunity. This implicates that not only patients administered systemic GCs, but also those who are given inhaled GCs can be more prone to infections, especially intracellular ones.

5. Conclusions

In conclusion, the research reported above showed that there was a CD8⁺ T cell response in the course of a mouse model allergic asthma that manifested itself by proliferation of these cells and their recruitment into the lower respiratory tract. Inhaled and systemic GC treatment fully prevented the said response. This strongly suggests that one of the elements composing the anti-asthmatic efficacy of inhaled and systemic GCs can be the inhibition of the effector CD8⁺ T cell response which accompanies the disease. The results suggest that the beneficial effects of GCs in asthma therapy are not mediated through generation or/and increased recruitment of Foxp3-expressing CD8⁺ Treg cells into the MLNs and lungs. Inhaled and systemic GCs produced comparable depletion of normal CD8⁺ T cells in the MLNs, HNLNs and PB, and this effect was, at least to some extent, a consequence of their proapoptotic effect on these cells. It cannot be excluded that inhaled GC treatment can induce depletion of CD8⁺ T cells in the lungs. The results strongly suggest that inhaled GC therapy might not be safer than maintenance treatment with systemic GCs in respect of the undesirable effects on CD8⁺ T cells residing within and outside the respiratory tract.

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 <https://doi.org/10.1016/j.intimp.2017.10.012>.

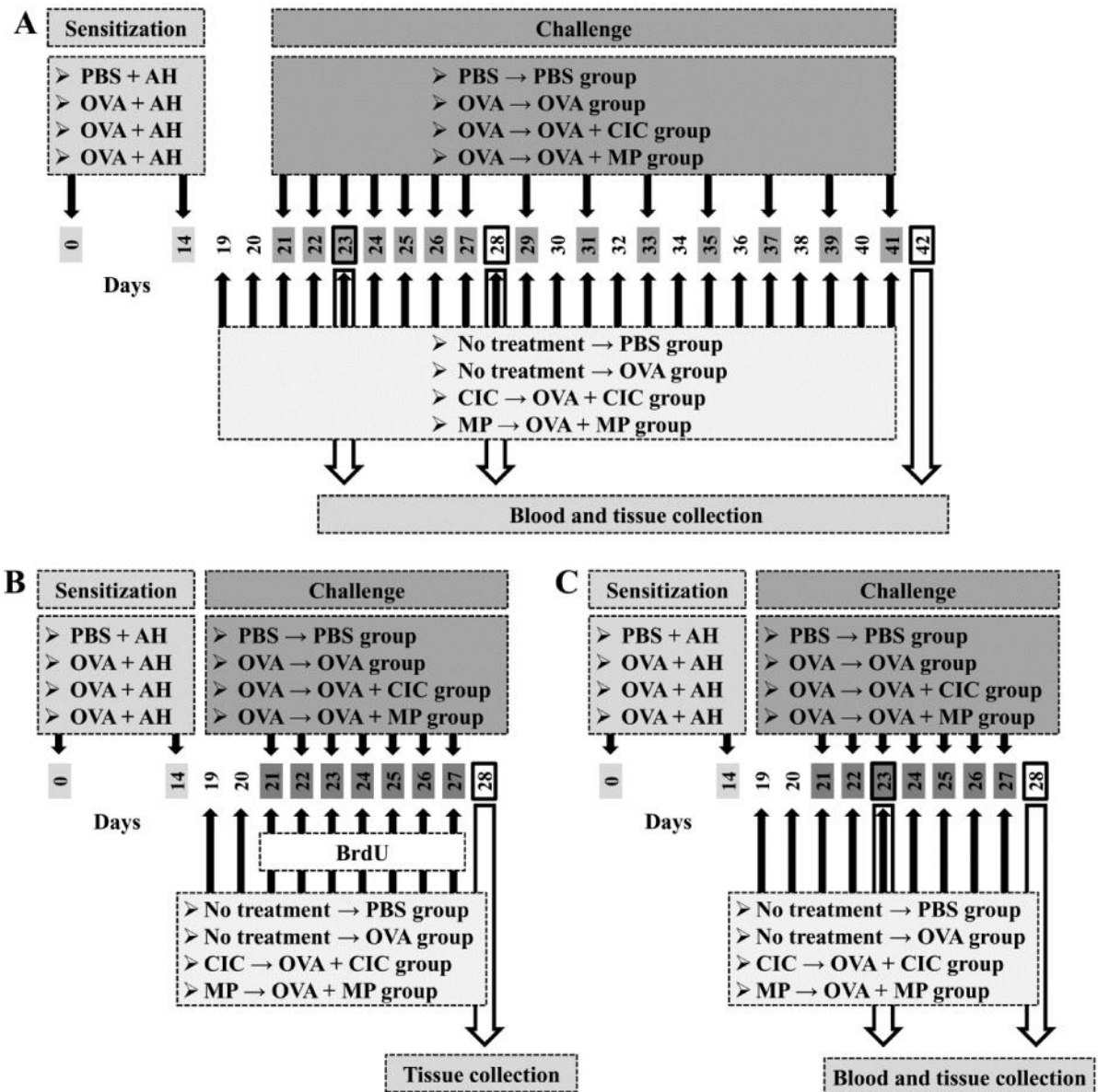
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Supplementary materials

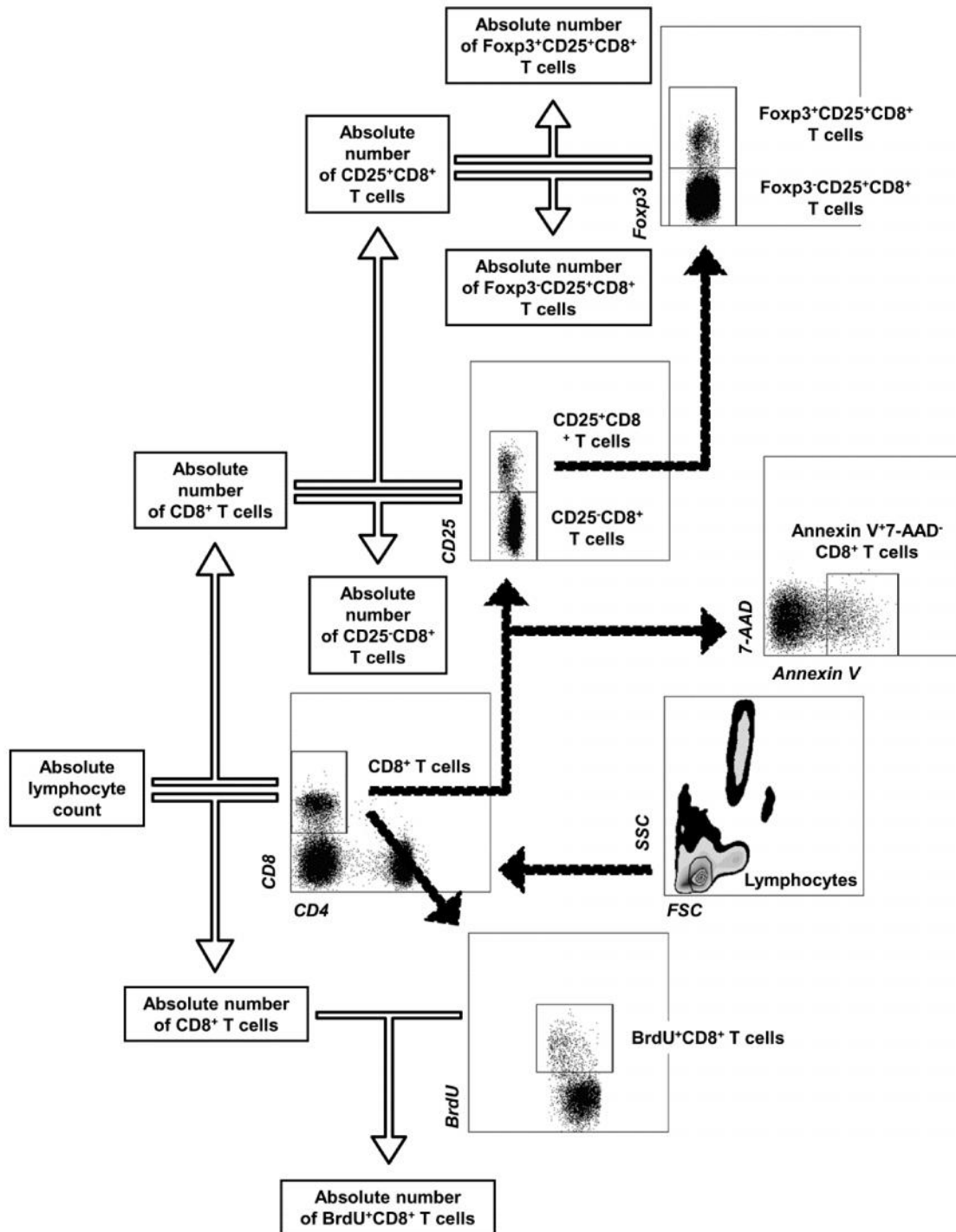
Inhaled glucocorticoid treatment prevents the response of CD8⁺ T cells in a mouse model of allergic asthma and causes their depletion outside the respiratory system

Monika Zuśka-Prot, Tomasz Maślanka



Supplementary figure 1. Schematic diagram of the study design. The main stages of the study included evaluation of effects of inhaled (Ciclesonide, CIC) and systemic (Methylprednisolone, MP) glucocorticoid treatment on: (a) the absolute counts of Foxp3⁺CD25⁺CD8⁺, Foxp3⁻CD25⁺CD8⁺ and CD25⁻CD8⁺ T cells in the mediastinal lymph

nodes (MLNs), lungs, head and neck LNs (HNLNs) and PB; this parameter was assessed on days 23, 28 and 42 of the experiment i.e. 2, 7 and 21 days after the initial OVA challenge (experiment A); (b) the number of proliferating CD8⁺ T cells in the MLNs and lungs; this effect was evaluated on day 28 of the experiment, i.e. 7 days after the initial OVA challenge (experiment B); (c) apoptosis of CD8⁺ T cells in the MLN, lungs, HNLNs and PB; effect of CIC and MP on apoptosis of these cells was assessed at two time points, i.e. on days 23 and 28 of the experiment, i.e. 2 and 7 days after the initial OVA challenge (experiment C). In each experiment mice were divided into four groups, namely PBS 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 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 the 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 and MP 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 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 (by asphyxiation with CO₂) 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).



Supplementary figure 2. 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 CD8 receptors. Relative to the presence of CD25 expression, the CD8⁺ T cell population was subdivided into CD25⁻ and CD25⁺ cell subsets. CD25⁺CD8⁺ T cells were analyzed for Foxp3

expression. CD8⁺ 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-AAD⁺) were considered to be a mixture of late apoptotic and primary necrotic cells. Moreover, CD8⁺ 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 μ l of blood or per tissue sample) were calculated using the dual platform method, as shown above.