

Impaired function of regulatory T cells in cord blood of children of allergic mothers

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Summary

Allergy is one of the most common diseases with constantly increasing incidence. The identification of prognostic markers pointing to increased risk of allergy development is of importance. Cord blood represents a suitable source of cells for searching for such prognostic markers. In our previous work, we described the increased reactivity of cord blood cells of newborns of allergic mothers in comparison to newborns of healthy mothers, which raised the question of whether or not this was due to the impaired function of regulatory T cells (T_{regs}) in high-risk children. Therefore, the proportion and functional properties of T_{regs} in cord blood of children of healthy and allergic mothers were estimated by flow cytometry. The proportion of T_{regs} [CD4⁺CD25^{high}CD127^{low}forkhead box protein 3 (FoxP3⁺)] in cord blood of children of allergic mothers tends to be higher while, in contrast, the median of fluorescence intensity of FoxP3 was increased significantly in the healthy group. Intracellular presence of regulatory cytokines interleukin (IL)-10 and transforming growth factor (TGF)-beta was also higher in T_{regs} of children of healthy mothers. Although we detected an increased proportion of T_{regs} in cord blood of children of allergic mothers, the functional indicators (intracellular presence of regulatory cytokines IL-10 and TGF-beta, median of fluorescence intensity of FoxP3) of those T_{regs} were lower in comparison to the healthy group. We can conclude that impaired function of T_{regs} in cord blood of children of allergic mothers could be compensated partially by their increased number. Insufficient function of T_{regs} could facilitate allergen sensitization in high-risk individuals after subsequent allergen encounter.

Keywords: allergy, cord blood, FoxP3, regulatory cytokines, T_{regs}

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Introduction

Allergy is one of the most common medical disorders with a constantly increasing incidence. One of the theories explaining such a tremendous increment of allergies is the hygiene hypothesis, which postulates that lower exposure to microbes, especially in developed countries, alters the development of the immune system, thus promoting allergy development in predisposed infants. There is a down-regulatory bias to T helper type 2 (Th2) immune responses in the prenatal period preventing undesirable interactions with antigenically different maternal constituents [1]. The establishment of a new immunological balance proceeds post-natally after encountering the external environment. Prevalent Th2 responses support allergy development;

Th1 and Th17 responses are important for anti-infection defence, but their exaggeration facilitates autoimmune reactions [2]. Therefore, very precise regulation preventing aberrant immune responses is important after birth. Regulatory T cells (T_{regs}) play an irreplaceable role in this fine tuning and limit pathological reactions, including allergy-associated Th2 responses.

There is a strong need to find early prognostic markers indicating increased risk of allergization. The finding of such a prognostic marker would make possible the introduction of preventive measures reducing allergy development, or at least lowering its clinical severity. Many authors have already tried to find some indications of future allergy development in cord blood. The responsiveness of cord blood cells of high- and low-risk children to allergens was followed [3,4],

and polyclonal G⁺/G⁻ bacteria stimulation [5–8] was tested. The proportion of both Th1 and Th2 cytokines in cord blood of high- and low-risk infants was tested [9–12]. Other researchers considered immunoglobulin (Ig)E levels in cord blood sera as a possible prognostic marker [13,14]. None of these measures have been found to be reliable prognostic indicators. It is possible to conclude with Prescott that the only reliable marker in allergy risk evaluation is the allergy status of the mother [5].

Several studies have tried to correlate the proportion of T_{regs} with the clinical symptoms of allergy in adults; unfortunately, results have as yet been contradictory [15–20]. Flow cytometry is usually used in these studies. Conflicting results may reflect variation in gating strategies used, different T_{regs} markers tested and also different ethnic groups examined. All these inconsistencies, together with the low number of individuals included in some studies [21,22], have led to ambiguous conclusions. Studies concerning cord blood T_{regs} and allergy are somewhat limited [22,23]. Based on the available studies, we postulate that some functional insufficiency of T_{regs} could contribute to allergy development. We tested this hypothesis by analysing and comparing T_{regs} in cord blood of high-risk newborns (children of allergic mothers) and low-risk newborns (children of healthy mothers). Using flow cytometry, we compared the proportion of T_{regs} (percentage of T_{regs} in the CD4⁺ population) and their functional properties [median of fluorescence intensity (MFI) of forkhead box protein 3 (FoxP3), interleukin (IL)-10 and transforming growth factor (TGF)-beta].

Materials and methods

Subjects

Healthy and allergic mothers with normal pregnancy and children delivered vaginally at full term in the Institute for the Care of Mother and Child in Prague, Czech Republic were included into the study. The diagnosis of allergy in mothers was based on the clinical manifestation of allergy persisting for longer than 24 months (allergy against respiratory and food allergens manifested by various individual combinations of hay fever, conjunctivitis, bronchitis, asthma, eczema and other allergic manifestations), monitoring by an allergist, positive skin prick tests or positive specific IgE antibodies and anti-allergic treatment before pregnancy. The study was approved by the Ethical Committee of the Institute for the Care of Mother and Child (Prague, Czech Republic) and was carried out with the written informed consent of the mothers.

A total of 153 maternal–child pairs were included in our study. Newborns were divided into two groups according to their mothers' allergy status: 77 children of healthy mothers (non-allergic) and 76 children of allergic mothers. Detailed description of the different types of allergy mothers involved in our study is summarized in Table 1.

Table 1. Detailed characteristics of mothers participating in this study.

| Allergy status | No. | Median age | | Max | Pollen | Mites | Dust | Cat dander | Food | Metal | Medicaments | Insects | Eczema | Others | 10–90 percentile | |
|----------------------|-----|------------|-----|-----|--------|-------|------|------------|------|-------|-------------|---------|--------|--------|------------------|---------------|
| | | Min | age | | | | | | | | | | | | IgE (r) IU/ml | IgE (f) IU/ml |
| Allergic mothers | 76 | 31 | 24 | 37 | 27 | 9 | 14 | 2 | 8 | 2 | 35 | 6 | 7 | 18 | 0.21–5.59 | 0.11–2.49 |
| Non-allergic mothers | 77 | 32 | 20 | 39 | | | | | | | | | | | 0.20–1.36 | 0.13–0.67 |

r: Specific IgE to a mixture of respiratory allergens; f: specific immunoglobulin (Ig)E to a mixture of food allergens.

Cord blood sampling

Typically, 10–20 ml of cord blood of children was collected in sterile heparinized tubes for cell analysis (T_{regs}). A questionnaire inquiring about the allergy status of the mother was completed during the stay at the Institute for the Care of Mother and Child.

T_{regs} ratio, FoxP3 staining

The proportion of T_{regs} was estimated in cord blood samples immediately after delivery. The whole cord blood was stained for T_{reg} cell surface markers using the following antibodies: CD4 fluorescein isothiocyanate (FITC), cat. no. 555346, CD25 phycoerythrin-cyanin 7 (PE-Cy7), cat. no. 557741 and CD127 Alexa 647, cat. no. 558598, all from Becton Dickinson (Franklin Lakes, NJ, USA). Lysis of erythrocytes was achieved by 12-min incubation with 3 ml of red blood cell (RBC) lysis buffer (cat. no. 00–4333) included in the human regulatory T cell whole blood staining kit (cat. no. 88–8996-40; eBioscience, San Diego, CA, USA). After centrifugation, followed by decantation of supernatant and washing (using 2 ml of flow staining buffer, cat. no. 00–4222, also included in the human regulatory T cell whole blood staining kit), cells were

permeabilized/fixed by incubation with 1 ml FoxP3 lysed whole blood (LWB) fixation/permeabilization working solution at 4°C for 1 h in the dark. After washing with 2 ml of flow staining buffer, cord blood samples were stained using the ‘gold standard’ marker for identifying T_{regs} with anti-human FoxP3 PE antibody, cat. no. 12-4776-41A (clone PCH101), also included in the human regulatory T cell whole blood staining kit (cat. no. 88–8996-40; eBioscience), for 30 min. After washing with 2 ml of flow staining buffer, the pellet was resuspended in 100 µl of flow staining buffer (no fixative added). Samples were examined immediately in order to prevent loss of fluorescence. The lymphocyte gate was set based on forward-scatter (FCS) and side-scatter (SSC) characteristics with doublets exclusion (FCS-A × FCS-H), then $CD4^+$ population was gated in the lymphocyte gate. Approximately 500 000 total events per sample were acquired for proper statistical evaluation of T_{reg} functional parameters. T_{regs} were analysed in the $CD4^+$ gate as an intercept of three subpopulations of $CD4^+$ lymphocytes using CD25, CD127 and FoxP3 markers ($CD25 \times CD127$, $CD25 \times FoxP3$, $CD127 \times FoxP3$). Detailed gating strategy for estimation of the T_{reg} ratio is shown in Fig. 1. Results are expressed as T_{reg} ratio and MFI.

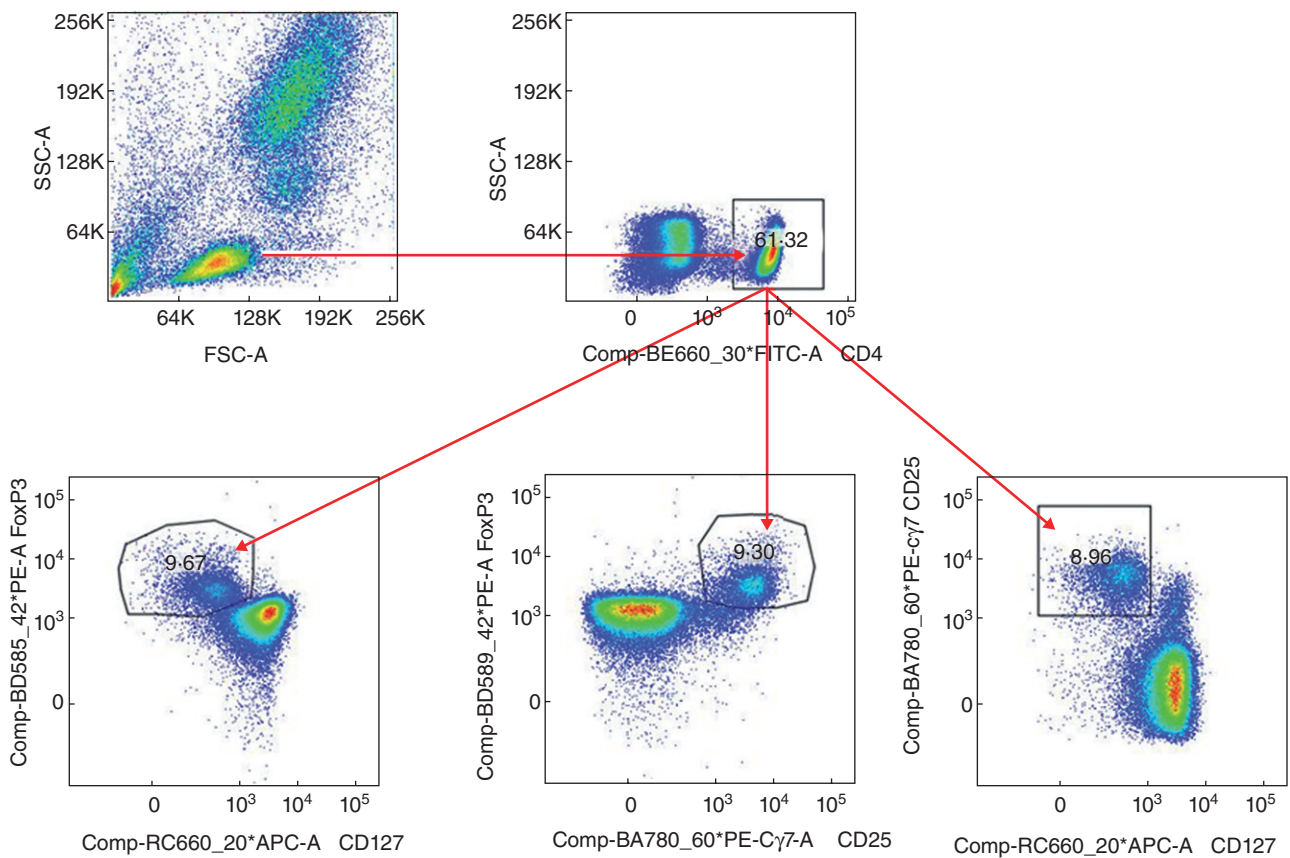


Fig. 1. Gating strategy of proportion of regulatory T cells (T_{regs}) in cord blood of children of healthy and allergic mothers. Expression of the proportion of T_{regs} in $CD4^+$ lymphocyte gate was considered as an intercept of three gates based on the combination of staining cell surface markers ($CD4$, $CD25$, $CD127$) and intracellular staining of transcription factor forkhead box protein 3 (FoxP3).

IL-10 and TGF-beta in T_{regs}

Regulatory cytokines were detected in non-stimulated cord blood cells. After red blood cell lysis and cell surface staining of CD4, CD25, CD127 (using the antibodies indicated above), intracellular staining of cytokines IL-10 (IL-10 PE, cat. no. 506804; BioLegend, San Diego, CA, USA) and TGF-beta [anti-human latency associated peptide (LAP) TGF-beta1 peridinin chlorophyll (PerCP)-Cy5.5, cat. no. 341803; BioLegend] was performed using fixation buffer, cat. no. 420801 and permeabilization wash buffer, cat. no. 421002 (both BioLegend) exactly according to the manufacturer's recommendations. For proper statistical evaluation, at least 100 000 total events were acquired per sample.

Data analysis and statistics

Flow cytometry data were acquired on a BD fluorescence activated cell sorter (FACS) Canto II instrument using BD FACS Diva version 6.1.2. software (Becton Dickinson). FlowJo 7.2.2. (TreeStar, Ashland, OR, USA) was used for data evaluation. Differences between groups were compared using the unpaired Student's *t*-test for data normally distributed (T_{reg} ratio, MFI of FoxP3); otherwise the non-parametric Mann-Whitney test was used (comparing proportion of IL-10⁺ T_{regs} and TGF-beta⁺ T_{regs}). Statistical and graphical analysis was performed in GraphPad Prism (Graph-Pad Software, La Jolla, CA, USA). Statistical significance was set at $P \leq 0.05$.

Results

The immunological characteristics of T_{regs} in cord blood of high-risk children (children of allergic mothers) and

low-risk children (children of healthy mothers) were compared. The proportion of T_{regs} was evaluated. To elucidate possible differences in functional properties of T_{regs}, MFI of FoxP3 and intracellular regulatory cytokines IL-10 and TGF-beta were tested. Differences in T_{reg} proportions and their functional properties were found between the groups.

T_{reg} ratio

Using our gating strategy (Fig. 1) and antibodies against CD4, CD25, CD127 and FoxP3, we did not find significant differences in the proportion of T_{regs} in the cord blood of children of healthy and allergic mothers, although the trend towards an increased number of T_{regs} in the CD4⁺ lymphocyte population from the allergic group was obvious ($P = 0.07$) (Fig. 2a). A significantly increased proportion of T_{regs} in cord blood of children of allergic mothers was observed when T_{regs} were considered only as CD4⁺CD25⁺ cells ($P = 0.0117$) (Fig. 2b). Different gating strategies together with using different T_{reg} markers may account for variation among the results of different research groups.

MFI of FoxP3

Transcription factor FoxP3 is considered to be a master marker for identifying T_{regs} [24] (as CD25 can be expressed on other activated CD4⁺ T lymphocytes and CD127 is present on various cell types). The values of MFI of FoxP3 in cord blood of children of allergic mothers followed an opposite trend to the proportion of T_{regs}. A significantly higher MFI of FoxP3 ($P = 0.0159$) in cord blood T_{regs} of children of healthy mothers was detected in comparison to children of allergic mothers (Fig. 3).

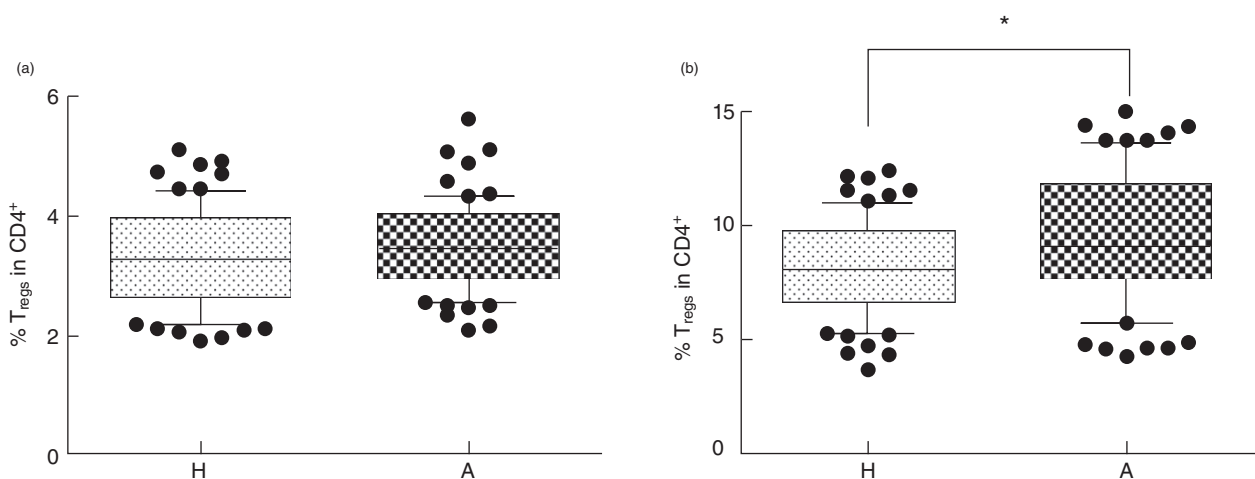


Fig. 2. Proportion of regulatory T cells (T_{regs}) in cord blood. (a) Four-colour flow cytometry analysis (intercept of CD4⁺CD25^{high}CD127^{low} and CD4⁺CD25^{high} forkhead box protein 3 (FoxP3⁺) and CD4⁺CD127^{low}FoxP3⁺). (b) Two-colour flow cytometry analysis (CD4⁺CD25⁺). H: proportion of T_{regs} in CD4⁺ lymphocytes in cord blood of children of healthy mothers (mean of 77 tested cord blood samples). A: proportion of T_{regs} in CD4⁺ lymphocytes in cord blood of children of allergic mothers (mean of 76 tested cord blood samples). * $P \leq 0.05$.

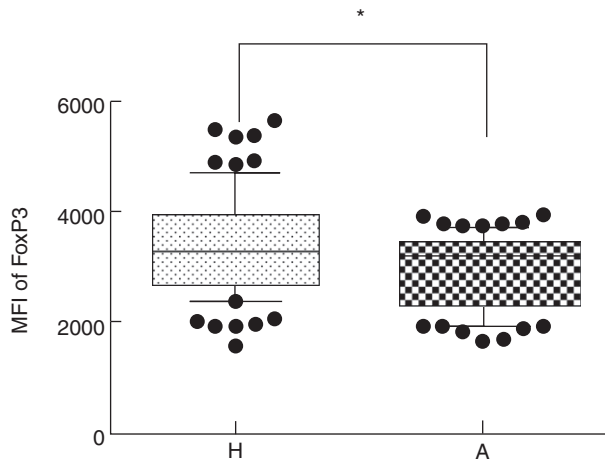


Fig. 3. Median of fluorescence intensity (MFI) of forkhead box protein 3 (FoxP3) in $CD4^+CD25^{high}CD127^{low}$ regulatory T cells (T_{regs}). H: MFI of FoxP3 in T_{regs} from cord blood of children of healthy mothers (mean of 77 tested cord blood samples). A: MFI of FoxP3 in T_{regs} from cord blood of children of allergic mothers (mean of 76 tested cord blood samples). * $P \leq 0.05$.

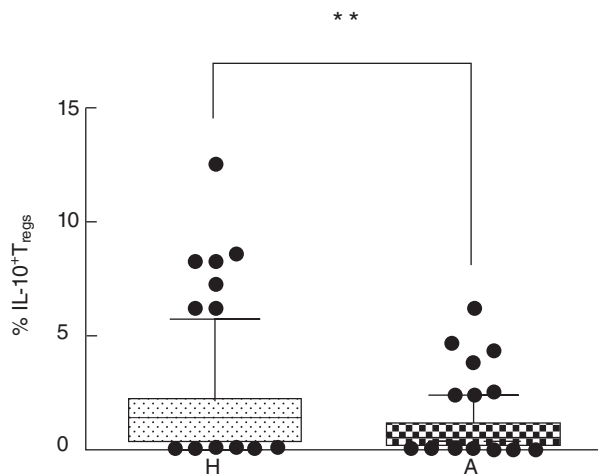


Fig. 4. Intracellular presence of IL-10 in regulatory T cells (T_{regs}). Percentage of interleukin (IL)-10-positive T_{regs} ($CD4^+CD25^{high}CD127^{low}$). H: percentage of IL-10⁺ T_{regs} in cord blood of children of healthy mothers (mean of 77 tested cord blood samples). A: percentage of IL-10⁺ T_{regs} in cord blood of children of allergic mothers (mean of 76 tested cord blood samples). ** $P \leq 0.001$.

Intracellular regulatory cytokines IL-10 and TGF-beta

To evaluate the possible differences in functional characteristics of T_{regs} , the presence of regulatory cytokines IL-10 and TGF-beta was estimated by intracellular staining. A significantly higher number of IL-10⁺ T_{regs} in cord blood of children of healthy mothers was detected in comparison to children of allergic mothers ($P = 0.0012$) (Fig. 4). Similarly, a significantly higher proportion of TGF-beta⁺ T_{regs} in cord blood of children of healthy mothers is documented in Fig. 5 ($P = 0.0174$).

Discussion

The importance of T_{regs} in immune regulations consists mainly in their role in induction of peripheral tolerance against autoantigens and harmless food and environmental antigens [25]. An insufficiency of T_{regs} can result in autoimmunity and allergy development [26–29]. We followed the status of newborn T_{regs} as a possible prognostic marker for future allergy manifestation. It is possible to assume that changes of immune regulation in allergy-prone infants can be evident prior to development of the clinical signs of allergy.

We found differences in immune characteristics of T_{regs} in the cord blood of children of allergic mothers in comparison to children of healthy mothers. T_{regs} were assessed on the basis of their cell surface markers ($CD4$, $CD25^{high}$ and $CD127^{low}$), typical transcription factor FoxP3 and intracellular regulatory cytokines IL-10 and TGF-beta. The lower presence of regulatory cytokines, together with decreased MFI of FoxP3 in T_{regs} in cord blood of children of allergic mothers, points to the lower functional efficiency of these cells [23,30]. Impaired function of T_{regs} in the cord blood of children of allergic mothers could be compensated partially by an increased number of T_{regs} in comparison with the healthy group.

We documented an increased proportion of $CD4^+CD25^{high}CD127^{low}FoxP3^+$ T_{regs} in children of allergic mothers. As indicated by Steinborn [23], FoxP3 is an important marker of regulatory cells reflecting their suppressor potency. When T_{regs} were detected only as $CD4^+CD25^+$ cells, their number was still higher. It is necessary to keep in mind that the above

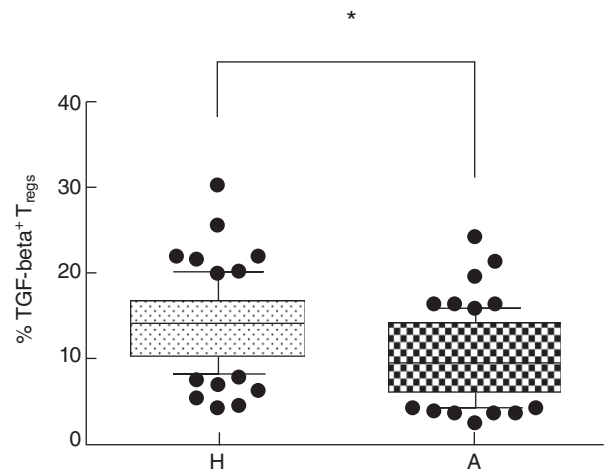


Fig. 5. Intracellular presence of transforming growth factor (TGF)-beta in regulatory T cells (T_{regs}). Percentage of TGF-beta-positive T_{regs} ($CD4^+CD25^{high}CD127^{low}$). H: percentage of TGF-beta⁺ T_{regs} in cord blood of children of healthy mothers (mean of 77 tested cord blood samples). A: percentage of TGF-beta⁺ T_{regs} in cord blood of children of allergic mothers (mean of 76 tested cord blood samples). * $P \leq 0.05$.

phenotype is characteristic not only for T_{regs}, but also for various subpopulations of activated T cells [31]. An increased proportion of the CD4⁺CD25⁺ subpopulation in cord blood of children of allergic mothers is in concordance with our previous observation of increased proliferation activity of both *in-vitro*-stimulated and non-stimulated cord blood cells of newborns of allergic mothers [32]. Discrimination between regulatory and activated T cells could be conducted on the basis of a recently described inverse correlation between CD127 and FoxP3 expression [33,34].

Regulatory cytokines IL-10 and TGF-beta are important effectors of T_{regs} [2,35,36]. Increased secretion of IL-10 (detected by ELISA) correlated with increased T_{regs} markers after stimulation of cord blood cells of children of healthy mothers, as reported by Schaub [37]. To the best of our knowledge, we are the first to report on the differences in the presence of intracellular IL-10 and TGF-beta between T_{regs} of children of healthy and allergic mothers. A lower proportion of T_{regs} producing IL-10 and TGF-beta in cord blood of children of allergic mothers (Figs 4 and 5) can signal a decreased predisposition to limiting the aberrant immune reaction to allergens in future, and can partially explain the increased proliferation activity of cord blood lymphocytes of children of allergic mothers mentioned above.

T_{regs} are a very heterogeneous population of cells and many methodological problems arise in the course of their study. Different gating strategies used for quantification of T_{regs} (CD4⁺CD25⁺ [38], CD4⁺CD25^{high} [30], CD4⁺CD25^{high}CD127^{low} [22], CD4⁺CD25^{high}FoxP3⁺ [39], CD4⁺CD25^{high}CD127^{low}FoxP3⁺ [40] or the gating we chose, based on the intercept of three different gates on CD4 subpopulation (as indicated in Fig. 1), can give quite different results leading to controversial conclusions. Furthermore, using different clones of FoxP3 antibodies could lead to different values of T_{reg} ratio [41,42]. Using different clones of FoxP3 antibodies allows the detection of different T_{reg} subpopulations. In our early experimental setting, we used two antibody clones (PCH101, eBioscience; and 259D/C7, Becton Dickinson) with appropriate buffers. A higher proportion of FoxP3⁺ T_{regs} was detected using PCH101, which was then used for further experiments. However, it is necessary to realize that the number of T_{regs} alone is not decisive for effective suppression function [43]. Functional analyses of T_{regs} are probably more informative. Further, it is necessary to keep in mind that not all lymphocytes exerting suppressor function express FoxP3 [44]. Another obstacle can be caused by cell isolation. Many studies analyse T_{regs} in peripheral blood after Ficoll-Paque separation. We compared the detection of T_{regs} in whole blood and in the population of isolated cord blood mononuclear cells (CBMC) – the results were similar, but the analyses obtained with the whole blood were more convincing and consistent and less time-consuming (data not shown).

We acknowledge some limitations of our study, namely the heterogeneity of mothers' allergies, but differentiation of

the children into subgroups according to different kinds of maternal allergy decreased the power of statistical analyses. Individual types of maternal allergies are listed in Table 1.

T_{regs} are thought to play an important role in immune regulations even during intrauterine life [7]. Increased numbers of T_{regs} in this period can be partially responsible for decreased neonatal immune responses. The function of T_{regs} is critical in the early postnatal period, when the tuning of the immature immune system takes place. The impairment of T_{regs} could be the underlying mechanism contributing to heightened allergy development in predisposed children.

Our proof of decreased functionality of T_{regs} in cord blood of children of allergic mothers is in full agreement with the work of Prescott [22], who tested the immune function of neonatal CD4⁺CD25⁺CD127^{low/-} T_{regs}. However, both Prescott [22] and Schaub [30] did not find significant differences in transcription factor FoxP3 between high- and low-risk infants, whereas other studies pointed to decreased function of T_{regs} based on the lower presence of FoxP3 (MFI) [23]. This could be explained either by low numbers of individuals included [22] or by different methods used for the quantification of FoxP3. Quantitative PCR (qPCR) was often used for the detection of FoxP3 gene expression [22,30]. Conversely, we exploited flow cytometry for FoxP3 protein detection. Schaub [30] suggests that the mRNA level of FoxP3 in T_{regs} is not regulated differently in dependence on maternal atopy. Nevertheless, the same group observed quantitatively and qualitatively increased T_{regs} in the cord blood of children of farming mothers whose children were postulated to be low-risk individuals for allergy development [7].

It is believed that lower exposure to non-pathogenic microbes together with reduced regulatory T function early in life could lead to Th1/Th2 imbalance, increasing the risk of allergy development [3]. The relationship between immune function of cord blood T_{regs} and allergy development requires further detailed studies. Our next intention is to follow the possible future allergy status of children participating in this study and to correlate this with the proportion and functional characteristics of T_{regs} in their cord blood. It could allow us to assess the value of T_{reg} characteristics as prognostic markers of allergy development. It would be of importance to include other recently described markers characterizing various subsets of T_{regs} in further studies (e.g. transcription factor Helios differentiating between nT_{regs} and iT_{regs}). Until now, no work has been focused on the presence of Helios in cord blood T_{regs}. Studies [7,43] suggest using the T_{reg}-specific demethylated region (TSDR), which seems to be a good qualitative marker indicative of functional activity. In fact, Hinz *et al.* [45] described a decreased proportion of T_{regs} characterized by TSDR in cord blood of children who develop allergy. Further studies characterizing T_{regs} by currently described T_{reg} markers are needed to assess the suitability of these markers to serve as prognostic indicators of functional impairment of T_{regs}.

In conclusion, our study points to the decreased immunological capacity of T_{regs} in the cord blood of children of allergic mothers in comparison to healthy mothers. Insufficient T_{reg} function can facilitate allergy induction in predisposed children. Long-term monitoring of children at risk is necessary for assessing the significance of the prognostic value of T_{reg} insufficiency at birth for future allergy development.

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