

Differences in circulating dendritic cell subtypes in cord blood and peripheral blood of healthy and allergic children

M. M. Hagendorens*†, D. G. Ebo†, A. J. Schuerwegh†, A. Huybrechts*, H. P. Van Bever*, C. H. Bridts†, L. S. De Clerck† and W. J. Stevens†

Departments of *Pediatrics and †Immunology, University of Antwerp, Antwerp, Belgium

Summary

Background Different types of circulating dendritic cells have been described. Dendritic cells influence differentiation of naive T lymphocytes into T helper type 1 (Th1) and Th2 effector cells.

Objective The purpose of this study was to evaluate the number of circulating DC subtypes in peripheral blood of allergic and healthy children and in cord blood of neonates from allergic and non-allergic parents.

Methods Circulating dendritic cells were flow cytometrically identified in whole blood samples as lineage (CD3, CD14, CD16, CD19, CD20, CD56) negative, CD34 negative and HLA-DR-positive cells. According to the expression of CD123 and CD11c, different DC subtypes were identified.

Results Apart from DC1 (CD11c⁺ CD123^{dim+}) and DC2 (CD11c[−] CD123^{high+}), a third DC population was described with less differentiated phenotypic characteristics, namely CD11c[−] CD123^{dim+}, and therefore defined here as less differentiated DC (ldDC). These ldDC represented the major DC population in cord blood and showed an age-dependent decrease. The highest level of ldDC was detected in children with atopic dermatitis, whereas asthmatic children showed the lowest ldDC counts. Furthermore, high-dose inhaled corticosteroid treatment in asthmatic children was related to a decreased ldDC number. The number of circulating DC2 was significantly lower in allergic children, especially in asthmatics, compared to healthy children. In cord blood, no differences in DC subtypes were detectable between neonates at low and high risk for allergic disorders.

Conclusion These results indicate that, apart from DC1 and DC2, a third population of dendritic cells, identified as CD11c[−] CD123^{dim+} cells and defined as less differentiated DC, must be considered in the evaluation of circulating DC. Furthermore, DC2 counts were decreased in allergic children, especially in asthmatics, which might be the consequence of an increased recruitment to the target organs.

Keywords allergy, asthma, atopic dermatitis, children, cord blood, dendritic cells, flow cytometry

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Introduction

Dendritic cells (DC) have an important modulatory role in the human immune system, as they are potent antigen-presenting cells. Located at all sites of possible antigen entry, they are responsible for antigen uptake, processing and presentation to T cells [1–4].

While migrating to the regional lymph nodes, DC become functionally mature and are then able to initiate immune responses in naive T cells [5]. Dendritic cells have been recognized to play a pivotal role in the regulation of T cell-dependent immune responses to antigens, mediated by their high expression of co-stimulatory molecules and production of regulatory cytokines [3, 6–8]. Two subtypes of DC have been identified, myeloid and lymphoid DC, with a different influence on naive

T cells [9, 10]. Myeloid DC (or DC1) express markers such as CD11c and CD33 and induce T helper type 1 (Th1) cell differentiation [11, 12], whereas lymphoid DC (or DC2) are highly positive for CD123, negative for CD11c and CD33 and induce Th2 effector cell development [13, 14]. Recently, a regulatory role of DC in allergic disorders was emphasized in clinical studies in adults [15–22]. To our knowledge, at present no data on circulating DC in allergic children are available.

In this study, DC subpopulations were quantified in cord blood and peripheral blood of healthy and allergic children, using a flow cytometric technique.

Materials and methods

Cord blood samples

Eighteen cord blood samples were randomly collected from term neonates, born after uncomplicated pregnancies (Table 1). A validated questionnaire for atopic diseases was

Correspondence: W. J. Stevens, Department of Immunology, University of Antwerp, Universiteitsplein 1, 2610 Antwerpen, Belgium.
E-mail: wim.stevens@ua.ac.be

used to assess the parental atopic status [23]. Ten neonates were defined to be at high risk to develop allergic diseases because at least one of the parents had a documented history of allergic asthma, rhino-conjunctivitis and/or atopic dermatitis. The eight remaining neonates were considered at low risk for atopy.

Healthy and atopic children

Twenty-nine atopic children were studied (Table 1). The majority of children ($n = 15$) suffered from allergic respiratory disease (asthma and/or rhinitis). Seven children had isolated mild to moderate atopic dermatitis, seven other children had combined atopic dermatitis and asthma. All patients showed an elevated specific IgE to at least one of the following allergens: *Dermatophagoides pteronyssinus*, timothy grass, birch, mugwort, cat, dog, egg's white or cow's milk (CAP systems, Pharmacia Diagnostics, Brussels, Belgium). The majority of asthmatic children (20/22) were treated with inhaled corticosteroids. β_2 -Agonists were used as rescue medication. Allergic rhinoconjunctivitis was treated with antihistamines and/or nasal corticosteroids. When indicated, topical corticosteroids were applied in case of atopic dermatitis. The median cumulative (nasal and/or inhaled) daily dose of corticosteroids in children with allergic asthma and/or rhinitis was 800 μ g beclomethasone dipropionate equivalent (range 100–2000 μ g); median forced expiratory volume of 1 s (FEV1) was 2.22 L (range 1.07–3.84 L) and 99% predicted (71–117%). According to the cumulative median daily dose of corticosteroids, children suffering from isolated respiratory allergy ($n = 15$) were subdivided into low-dose (< 800 μ g beclomethasone dipropionate; $n = 7$) and high-dose groups (≥ 800 μ g beclomethasone dipropionate; $n = 8$); there was no topical dermal corticosteroid use in this group of atopics. None of the children received systemic corticosteroids during the 6 months prior to inclusion. At the moment of blood sampling, children were free of any sign of acute respiratory distress and/or secondary infection of the skin lesions.

An age-matched group of 24 healthy children without history of allergic symptoms and with negative specific IgE to the different allergens tested (see above), served as control group (Table 1). These children were free of infection and medication during the month preceding the blood sampling.

The parents of all participating children gave their informed consent. The ethics committee of the University of Antwerp approved the study (file number A02-042).

Flow cytometrical identification of dendritic cells

DC were identified in heparinized whole-blood samples (Greiner Bio-One, Frickenhausen, Germany) using a combination of

fluorochrome-conjugated monoclonal antibodies to different membrane markers [24]. Reagents were obtained from BD Biosciences (Erembodegem, Belgium): 20 μ L lineage cocktail 1-fluorescein isothiocyanate (Lin1-FITC), containing a mixture of anti-CD3, CD14, CD16, CD56, CD19 and CD20 antibodies, 5 μ L anti-CD34-FITC, 5 μ L anti-CD123-PE, 5 μ L anti-CD11c-APC and 10 μ L anti-HLA-DR-PerCP were incubated with 100 μ L whole blood for 15 min at room temperature in the dark. Cells were fixed and lysed during 20 min at room temperature using FACS lysing solution (BD Bioscience). Cells were spun at 200 g for 10 min and resuspended in 0.5 mL phosphate-buffered saline, containing 0.1% Na₃.

Data acquisition was performed on a 4-colour FACScalibur (BD Bioscience) using a well-defined gating strategy in CellQuest Pro software. At least 5×10^5 cells were measured in order to obtain a minimum of 1000 HLA-DR-positive DC.

Three different cell populations could be identified: DC1 (or myeloid DC) were CD11c⁺ CD123^{dim+}, DC2 (or lymphoid DC) were CD11c⁺ CD123^{high+}. However, as shown in Fig. 1, apart from DC1 and DC2, a third population of lineage⁺ CD34⁺ HLA-DR⁺ cells could be distinguished, as well in cord blood as in peripheral blood. These cells were characterized phenotypically as CD11c⁺ CD123^{dim+} and therefore considered as less differentiated DC (ldDC). Consequently, the total number of DC was calculated as the sum of DC1, DC2 and ldDC.

Results were expressed as percentages of total white blood cells and absolute values were calculated, using white blood cell counts.

Statistics

Results are expressed as median and range. Differences between groups were analysed using Kruskal–Wallis and Mann–Whitney test. Correlations were evaluated with the Spearman's rank correlation coefficient. A P -value < 0.05 was considered significant.

Results

Three subtypes of circulating DC: DC1, DC2 and ldDC

In both cord blood and peripheral blood, three subsets of lineage⁺ CD34⁺ HLA-DR⁺ cells could be identified, namely the classical DC1 and DC2 and the newly described less differentiated DC (ldDC; Fig. 2).

CD11c⁺ CD123^{dim+} DC (ldDC)

In cord blood, ldDC represented the major DC subtype. The median ldDC count in cord blood was comparable between

Table 1. Subject characteristics

	Healthy children	Allergic children	Cord blood
Number	24	29	18
Age (years)	7.5 (1–15)	10.0 (1–18)	0
Sex (M/F)	15/9	23/6	7/11
White blood cells ($10^6/L$)	6950 (4900–12 500)	6500 (3500–16 600)	12 950 (9300–16 700)
Eosinophils ($10^6/L$)	280 (70–860)	440 (60–1070)	ND
Basophils ($10^6/L$)	40 (0–110)	40 (0–230)	ND
Total IgE (kU/L)	33 (1–574)	536 (8–5350)	ND

Data expressed as median values and range (in brackets). ND, not done.

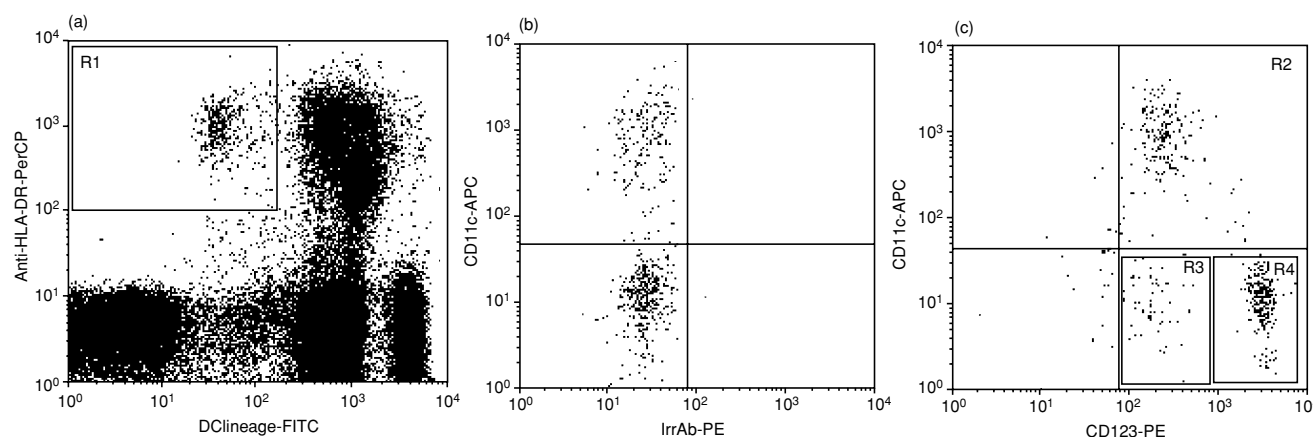


Fig. 1. Gating strategy for identification of dendritic subtypes. Mononuclear cells were first gated out according to the forward and sideward scatter. (a) Dendritic cells (R1) were characterized as cells negative for lineage markers (DClineage-FITC) on T lymphocytes (CD3), B-lymphocytes (CD19, CD20), monocytes (CD14), neutrophils (CD16), natural killer cells (CD16, CD56) and haematopoietic progenitor cells (CD34) and positive for HLA-DR-PerCP. (b) All gated DC (R1) incubated with CD11c-APC and an irrelevant isotype control antibody (IrrAb-PE). (c) After gating DC (R1) and incubation with CD11c-APC and CD123-PE, three different cell populations were distinguished on a CD123-PE/CD11c-APC plot: DC1 were CD11c⁺ CD123^{dim+} (R2), DC2 are CD11c⁺ CD123^{high+} (R4) and CD11c⁻ CD123^{dim+} cells (R3) were considered as less differentiated DC (IdDC).

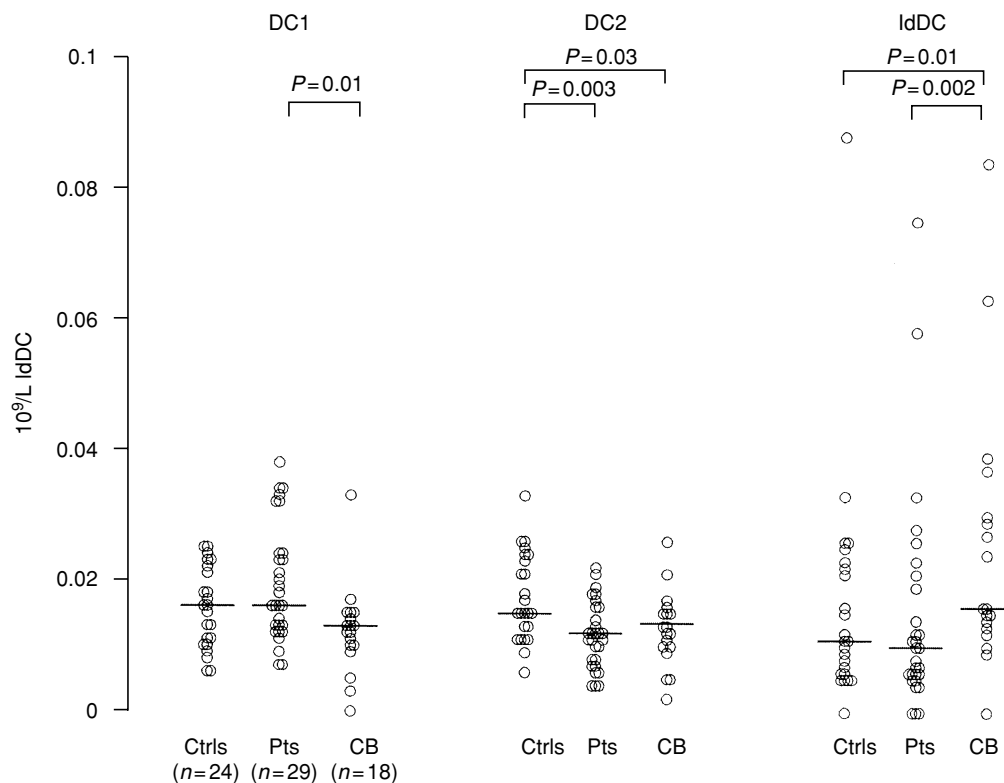


Fig. 2. Number of circulating DC1, DC2 and IdDC in peripheral blood of healthy controls (ctrls) and allergic patients (pts) and in cord blood (CB). The horizontal bar indicates the median value.

neonates at low and high risk for atopic disorders (data not shown).

In peripheral blood, the number of IdDC was the lowest of the different DC subtypes and did not differ between healthy children and the pooled group of allergic children. However, the number of IdDC showed an age-dependent decrease, particularly in healthy children ($r = -0.57$; $P = 0.003$), but also in allergic children ($r = -0.46$; $P = 0.01$; Figs 3a and b).

Furthermore, the highest level of IdDC in peripheral blood was detected in children with isolated atopic dermatitis, whereas asthmatic children showed the lowest IdDC counts ($P = 0.02$); the number of IdDC in healthy children was intermediate and did not significantly differ from the allergic groups (Fig. 4).

Within the group of asthmatic children, the possible influence of the dose of inhaled corticosteroids was evaluated. Asthmatic

children receiving high-dose corticosteroid treatment showed a statistically significant decreased IdDC count as compared to healthy controls [median, respectively, $0.005 \times 10^9/\text{L}$ (range 0.001–0.012) vs. $0.011 \times 10^9/\text{L}$ (range 0.001–0.088), $P=0.01$]

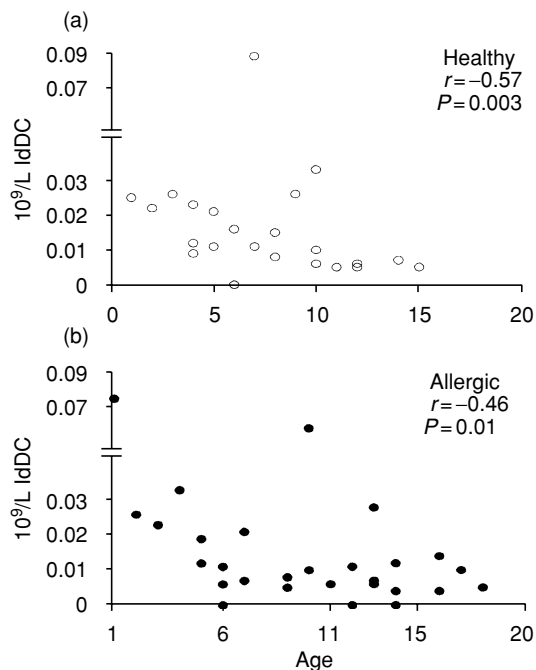


Fig. 3. Age-related decrease of IdDC in healthy (a) and allergic (b) children.

and a lower IdDC count as compared to children treated with a low dose of inhalant corticosteroids [$0.011 \times 10^9/\text{L}$ (range 0.01–0.028), $P=0.07$]. Asthmatic children with a high-dose regimen of inhalant steroids were significantly older than healthy controls ($P=0.017$), but were comparable in age compared to children with low-dose treatment.

DC1 subtype

In peripheral blood of both healthy and allergic children, DC1 represented the major DC population. Peripheral blood DC1 counts did not differ between healthy and allergic children. However, in allergic children, a positive correlation between the number of DC1 and eosinophil count was found ($r=0.64$; $P=0.0002$).

The number of DC1 in cord blood was significantly lower compared to peripheral blood of allergic children ($P=0.01$), but comparable to DC1 counts in healthy children. The number of DC1 on cord blood was not related to the allergic status of the parents.

No age-related changes in the number of circulating DC1 could be detected.

DC2 subtype

DC2 counts in cord blood were significantly lower compared to peripheral blood of healthy children ($P=0.03$). Again, no differences in DC2 counts were detected between neonates at low and high risk for allergic disorders. However, allergic children showed a significantly decreased DC2 count compared to healthy controls ($P=0.003$). Consequently, the ratio of DC2/DC1 was significantly lower in the allergic group [median value

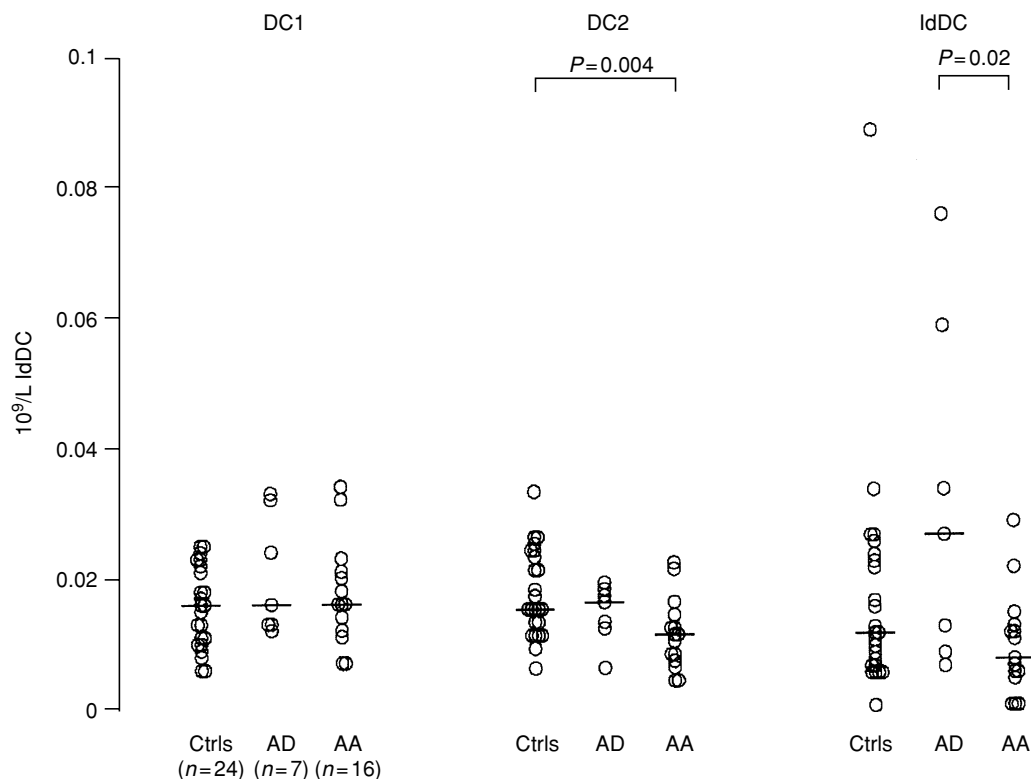


Fig. 4. Number of circulating DC1, DC2 and IdDC in peripheral blood of healthy controls (ctrls), compared to children with allergic asthma (AA) and atopic dermatitis (AD). The horizontal bar indicates the median value.

0.5 (range 0.3–2.0)] compared to the control group [median value 1.0 (range 0.5–4.0), $P = 0.0001$] and to cord blood values [median value 1.0 (range 0.5–2.0), $P = 0.0001$].

When allergic children with isolated asthma and atopic dermatitis were compared to healthy children, only DC2 counts in asthmatic children were significantly lower compared to healthy children ($P = 0.004$).

No age-related changes in the number of circulating DC2 could be detected.

Total number of circulating DC

The total number of DC in neonates, healthy and allergic children was comparable.

Discussion

Apart from their antigen-presenting capacity, DC are considered to play an important immunoregulatory role in T helper cell differentiation. DC1 and DC2 are implicated in Th1 and Th2 effector cell development, respectively [7, 10]. To assess the possible contribution of DC in the pathogenesis of allergic disorders in children, the number of circulating DC subtypes was flow cytometrically studied in cord blood of neonates and peripheral blood of healthy and allergic children.

A combination of monoclonal antibodies was used to identify DC, as lineage (CD3, CD14, CD16, CD19, CD20, CD56) negative (lineage⁻), CD34-negative (CD34⁻) and HLA-DR-positive (HLA-DR⁺) cells [10, 24]. Further characterization of DC subtypes was performed using the myeloid marker CD11c and the lymphoid marker CD123, i.e. the α -chain of the IL3-receptor [10–12]. Three DC subtypes could clearly be distinguished, as well in cord blood as in peripheral blood: DC1 (or myeloid DC) are CD11c⁺ CD123^{dim+} and DC2 (or lymphoid DC) are CD11c⁻ CD123^{high+}, but also a population of lineage⁻ CD34⁻ HLA-DR⁺ cells that was observed to be CD11c⁻ CD123^{dim+}. This cell population was presumed to be dendritic cells, based on the lack of expression of lineage markers and CD34, and the high expression of HLA-DR. Furthermore, considering the weak expression of CD123 and the absence of CD11c, we hypothesize that this DC population might represent a less differentiated DC subtype (ldDC). This presumption is further supported by additional experiments on isolated DC, showing that these ldDC weakly express BDCA-4 (data not shown); BDCA-4 and BDCA-2 were recently described as DC2 surface antigens [25]. The heterogeneity of lineage⁻ HLA-DR⁺ cells has been emphasized recently by MacDonald et al. describing five non-overlapping subsets, with CD11c⁺ CD16⁺ cells as a major population [26]. Furthermore, the functional characteristics of CD11c⁻ CD123^{dim+} ldDC remain to be elucidated.

These ldDC could be detected in all cord and peripheral blood samples. The increased number of ldDC in cord blood, compared to peripheral blood, is in line with other data on cord blood, where a higher count of immature cell types, such as naive T cells has been found [27]. Furthermore, as described for other immature cell types, ldDC counts decreased with age, particularly in healthy children. Age-dependent changes in circulating DC were reported in a recent study by Teig [28], who described a decrease of CD123⁺ DC, especially during the

first 5 years. However, no distinction was made between CD123^{dim+} and CD123^{high+} DC.

In a recent article, using the same methodology for identification of circulating DC in whole blood, Uchida [16] also showed a similar population of lineage⁻ CD34⁻ HLA-DR⁺ cells, missing definite DC1 or DC2 characteristics. However, this DC population was not discussed, as only DC1 and DC2 numbers were analysed in relation to allergy in adults. In addition, in human tonsils different lineage⁻ HLA-DR⁺ DC were described, including a CD11c⁻ CD123⁻ population, which might represent a similar DC subtype, with even less differentiated characteristics than the ldDC, as defined by us [29].

One might argue that the higher ldDC counts in atopic dermatitis as compared to healthy and asthmatic children might, at least partly, merely reflect an age effect. However, given the lower numbers of Th2-inducing DC2 cells in asthmatic children, additional explanatory mechanisms need to be considered. It could be hypothesized that these results mirror differences in recruitment of DC subtypes to the airways and to the skin. A complex network of chemokines, chemokine receptors and adhesion molecules are involved in the migration of many cell types, including DC [30]. The observed differences in ldDC and DC2 counts within the group of allergic children, comparing respiratory symptoms with atopic dermatitis, might consequently be explained by differences in chemokine production by residing tissue cells (airways vs. skin), in chemokine responsiveness of the circulating DC and/or in expression of cell adhesion molecules on endothelial cells of airway mucosa versus skin [31, 32].

Recruitment of circulating DC has been suggested in allergen challenge studies in allergic adults, but available data are contradictory. An accumulation of DC2 in nasal mucosa was shown after repeated allergen challenge in adults with allergic rhinitis [18]. In contrast, tissue accumulation of circulating DC1 to airways has been suggested in two recent papers in adult asthmatics, where, after short-term allergen challenge, a rapid decrease of circulating DC1, identified as CD33⁺ DC, was shown by Upham [17], whereas Jahnsen [19] described a rapid accumulation of airway myeloid DC.

Within the group of children with allergic respiratory diseases, DC counts were evaluated in relation to the total daily amount of inhaled corticosteroids (bronchial and/or nasal), which was considered as an indirect marker of disease severity. As ldDC counts increased from asthmatics receiving high-dose inhaled corticosteroids over the age-matched low-dose group up to the values in healthy children, it is anticipated that ldDC might be involved in mechanisms of respiratory disease severity. It seems unlikely that use of inhaled corticosteroids induces similar effects on circulating DC as systemic corticosteroids, as has been described by Shodell, who reported a reduction in circulating DC2 numbers after oral steroid administration [33]. However, a corticosteroid effect cannot totally be ruled out.

To our knowledge, no comparable studies on DC in paediatric patient groups are available. In addition, a few studies evaluating involvement of DC in allergic disorders in adults show contradictory results. Uchida et al. [16] described an increased number of DC2 (CD11c⁻ CD123⁺) in peripheral blood of allergic adults, suffering from asthma or atopic dermatitis. A report by Reider et al. [15], where DC were identified only according to absence or presence of CD123, could not detect significant differences in DC counts (DC1, DC2 and

total DC) in peripheral blood of adults with allergic rhinoconjunctivitis or atopic dermatitis compared to healthy controls. It has to be acknowledged that some of the described discrepancies between studies might also be attributable to methodological differences, such as gating strategy and antibodies used, and to the characteristics of the study population.

No differences in cord blood DC subtypes were detectable between neonates at low and high risk for allergic disorders, suggesting that the parental allergic status did not influence the number of circulating DC in cord blood. These results might be explained by the absence of allergic disease activity in the neonates studied, which was also noticed in a previous study in our laboratory, where no differences in either T lymphocyte subtypes nor intracellular cytokine production could be detected [34].

In conclusion, our data demonstrate that, apart from DC1 and DC2, a third population of circulating DC, CD123^{dim+}CD11c⁻ cells, designated ldDC, must be considered in the evaluation of circulating DC. These ldDC represent the major DC population in cord blood, whereas their counts gradually decrease with age. Moreover, it is suggested that ldDC might be involved in atopic dermatitis and in more severe allergic respiratory disease in children. Furthermore, it was shown that allergic diseases in children, especially asthma, are related to a decreased DC2 count, which are presumed to reside in the target organs.

As available data on the role of different DC subsets in allergic disorders show conflicting results, additional functional studies on circulating DC will be required; especially the role of ldDC needs to be examined by additional *in vivo* and *in vitro* studies.

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