

Original article

Comparative immunophenotyping of monocytes from symptomatic and asymptomatic atopic individuals

Background: Allergy has at least two components – a genetic predisposition referred to as atopy and the progress from an atopic state to clinically apparent disease. Peripheral blood monocytes are circulating myeloid precursors of antigen-presenting cells. The expression of cell surface proteins on monocytes may therefore witness the disease status and affect the development of allergic disease.

Methods: Monocytes were isolated from atopic individuals with seasonal allergic rhinitis ($n = 10$), from atopic individuals sensitized to aeroallergens but without any signs of acute disease ($n = 11$), and from healthy nonatopic donors ($n = 21$). Detailed comparative phenotypic analysis of $CD14^+$ and $Fc\epsilon RI^+ CD14^+$ monocytes was performed by flow cytometry.

Results: $CD14^+$ monocytes from symptomatic atopic donors showed a significant increase in the cell surface intensity of the integrin adhesion molecule CD11c over monocytes from asymptomatic atopic and nonatopic donors. Asymptomatic atopic individuals showed significantly enhanced expression of $Fc\epsilon RI$ on the $CD14^{high}CD16^{dim}$ monocyte subset compared with this subset from symptomatic atopic and nonatopic donors.

Conclusion: The increase in monocyte surface intensity of the adhesion molecule CD11c may be involved in the manifestation of allergic disease. $Fc\epsilon RI$ on $CD14^{high}CD16^{dim}$ monocytes of asymptomatic atopic donors may be of functional importance for the maintenance of clinical unresponsiveness toward allergens.

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Atopic individuals show increased serum levels of total and specific IgE to allergens such as house dust mites, pollen, food allergens, or animal danders. Most often, these individuals have a family background of atopic diseases such as allergic rhinitis (AR), allergic asthma or atopic dermatitis (1). Antigen-presenting cells (APCs) from atopic individuals show genetically determined abnormalities which may play an important role in the pathophysiology of atopic disorders (2). For example, the intensity of surface expression of the high-affinity receptor for IgE, $Fc\epsilon RI$, on APCs is positively associated with the atopic status of the individual (3, 4). Functionally, $Fc\epsilon RI$ mediates efficient IgE-dependent allergen uptake and presentation to T cells (5). These findings may add to a better understanding of the pathophysiology of IgE-mediated APC functions. As allergen sensitization does not necessarily lead to the clinical manifestation of allergic disease, different regulatory responses may take place in atopic individuals after allergen challenge. In addition to genetically determined characteristics of atopy, atopic individuals can be divided into symptomatic atopic individuals (SA) and asymptomatic atopic indi-

viduals (AA). AA show specific IgE toward various environmental allergens but do not present clinical signs of allergic disease. In this case, the presence of specific IgE probably indicates acquired sensitization to allergens on an atopic background or is a marker of possible future allergic manifestation.

The prerequisite for allergen-specific activation of T cells is recognition of major histocompatibility complex-bound antigenic, allergen-derived peptides expressed on APCs by the T-cell receptor complex (6). Costimulatory signals as well as adhesive interactions from APCs, which enable intimate intercellular contacts critically determine whether an effective stimulatory signal is delivered to T cells (7, 8). Monocytes play an important role as APCs in the initiation of the specific immune response (9). Moreover, monocytes are direct precursors for $CD11c^+$ DCs, one of the most important mediators of allergic inflammation (10). The expression of specific antigens and costimulatory molecules on monocytes is likely triggered by a combination of several factors. Allergen-challenged microenvironments may evoke direct activation of monocytes and lead to phenotypical and functional changes in these cells.

The two main CD14⁺ monocyte populations in humans, i.e. the CD14^{high}CD16^{neg} 'classical' monocytes and the CD14^{dim}CD16^{high} monocytes could be functionally and phenotypically correlated with two major murine monocyte subsets with distinct migratory properties (11). According to this model, CD14^{high}CD16^{neg} monocytes home to sites of inflammation whereas CD14^{dim}CD16^{high} monocytes give rise to resident, specialized cell types in various tissues. The respective contributions of these populations to the initiation of allergy are unclear. FcεRI⁺ monocytes may be direct precursors for FcεRI⁺ DC that are recruited into sites of allergic inflammation. Therefore, allocation of FcεRI⁺ monocytes to these subpopulations and possible differences thereof between atopic and nonatopic individuals may help to clarify the development of allergic disease.

Analysis of differences between clinically symptomatic and asymptomatic individuals is a promising approach to delineate factors that are causal to allergic inflammation. To determine a possible role for specific surface antigens on monocytes in the manifestation of allergic disease, CD14⁺ monocytes and FcεRI⁺CD14⁺ monocytes from symptomatic (SA), asymptomatic (AA) and from nonatopic (NA) individuals were subjected to detailed phenotypical analysis.

Material and methods

Reagents

The following monoclonal antibodies (mAb) were used for flow cytometric analysis: mAb 22E7 recognizes the FcεRIα-chain and was a kind gift of J. Kochan (Hoffmann-La Roche Diagnostics, Nutley, NJ). An unlabeled mAb against HLA-DR (L243) was kindly provided by N. Koch (Department of Zoophysiology, University of Bonn, Germany). FITC-labeled F(ab')₂ of goat-anti-mouse Ab (GaM/FITC) were from Jackson ImmunoResearch Laboratories (West Grove, PA). Unlabeled anti-CD123 (IL-3 receptor α-chain), anti-CD40, anti-CD64, anti-CD86, PE-labeled mAb to CD86, CD80, CD14, IgG2b, Percip-labeled mAb to IgG2a, CD14, Cy3-labeled matched control mAb and anti-CD16-Cy3 were obtained from Becton Dickinson Immunocytometry systems (San Jose, CA). Unlabeled CD23, CD16 (3G8), CD32, CD83, CD11a, CD11b, CD11c and PE-labeled CD40 were from Immunotech (Marseille, France).

Donors and diagnostic criteria

Donors were volunteers and were selected in accordance with the local ethics committee. Blood samples of all donors were taken during the pollen season. The following participants were recruited: SA individuals with seasonal AR (*n* = 10), AA individuals (*n* = 11), and nonatopic individuals (*n* = 21).

Symptomatic atopic individuals with seasonal AR were enclosed into the study if they (i) presented with typical signs of seasonal AR; (ii) showed specific IgE (>0.7 kU/l, CAP-Class 2) toward the relevant aeroallergen(s) with or without elevated total serum IgE levels (>100 kU/l); (iii) did not present with any additional atopic disease such as allergic conjunctivitis, allergic asthma or atopic dermatitis at least 5 years before the study and at the time of

investigation. None of the patients had been previously treated with immunotherapy or was receiving steroid or antihistamine treatment. AAs were selected if (i) they showed elevated specific IgE (>0.7 kU/l, CAP-Class 2) toward aeroallergens such as pollen, with or without an increased total IgE serum level (>100 kU/l) and with or without sensitivity to other common environmental allergens from house dust mites, food, or animal dander; (ii) donors did not present with the clinical manifestation of either atopic disease such as allergic conjunctivitis, AR, allergic asthma, or AD at the time of the study and at least 5 years before. Nonatopics (NA) (i) never presented with either atopic disease; (ii) they did not show elevated serum IgE levels (<100 kU/l) nor specific IgE (<0.7 kU/l, ≤CAP-Class 1) toward common environmental allergens such as allergens from pollen, house dust mites, food, or animal dander.

Monocyte isolation

Monocytes were isolated from peripheral blood with a density-gradient protocol using Nycoprep (Nycomed, Oslo, Norway). Briefly, red blood cells were separated from plasma by sedimentation from EDTA blood with one-tenth (w/v) 6% dextran 500 in 0.9% NaCl. Plasma was layered over Nycoprep and centrifuged for 20 min at 600 *g*. After separation, the interphase and upper part of the Nycoprep were collected and washed three times. Monocyte isolation was confirmed by CD14 expression and was >90%.

Immunostaining

For two-color analysis of monocytes, the cells were incubated at 4°C with the first antibody or an isotype control Ab at 0.5 μg/ml for 20 min. The cells were washed in PBS + 1% FCS + 0.1% sodium azide and incubated at 4°C with goat-anti-mouse-FITC (GaM/FITC) for 15 min. After washing, GaM/FITC was blocked with normal mouse serum for 15 min, washed, and the cells were counterstained at 4°C with anti-CD14 mAb and 7-amino-actinomycin D (7-AAD) (1 μg/ml) for 20 min. For three-color analysis, monocytes were first incubated with 22E7 mAb (anti-FcεRIα-chain) or an isotype control. After washing, GaM/FITC was added for 15 min. Unspecific binding of GaM/FITC was blocked with mouse serum as above. Mouse-anti-human CD14-PerCip or CD14-PE was added for 20 min at 4°C. After washing, a third mAb was added (anti-human CD40-PE, anti-human CD80-PE, anti-human CD86-PE, or anti-human CD16-Cy3).

Flow cytometric analysis

A FACS Calibur cytometer (Becton Dickinson) was used to determine the surface intensity of various antigens on peripheral blood monocytes.

CD14⁺ monocytes (two-color analysis)

The vital and CD14⁺ monocyte population was gated by a combination of forward and side scatter (FSC/SSC) and CD14/ 7-AAD gate sets. Fluorescence intensities of various antigens were determined as the relative fluorescence index (rFI). The mean fluorescence intensity (MFI) for each antigen of the vital CD14⁺ population was determined. rFIs were assessed as follows:

$$rFI = \frac{[MFI(\text{antigen}) - MFI(\text{isotype control})]}{MFI(\text{isotype control})}$$

FcεRI⁺CD14⁺ monocytes (three-color analysis)

FcεRI was defined to be expressed on monocytes if >10% of CD14⁺ cells of a donor stained positive with the mAb 22E7 compared with the isotype control ($n = 10$ for the SA group; $n = 10$ for the AA group; $n = 11$ for the NA group).

For the analysis of FcεRI⁺ monocytes, the CD14⁺ population was gated as described above. To determine the expression intensity of FcεRI on monocyte subpopulations, CD16 antigen expression was determined in addition to FcεRI and CD14. In addition to the CD14 gate, another gate was set around CD16^{neg}, CD16^{dim} and CD16^{high} monocytes, respectively. The intensity of FcεRI on these monocyte subpopulations was expressed by determination of the rFI.

To assess costimulatory molecule expression on FcεRI⁺CD14⁺ monocytes, in addition to the CD14-gate, another gate was set around FcεRI⁺ monocytes. The expression intensity of CD40, CD80 and CD86 on these FcεRI⁺CD14⁺ monocytes was evaluated quantitatively by determination of the rFI.

Statistical analysis

The Mann–Whitney–Wilcoxon test was applied for statistical differences between groups using SPSS 11.0 for WINDOWS (SPSS, Chicago, IL). $P < 0.05$ was considered to indicate a significant difference between the groups.

Results

CD11c is strongly expressed on CD14⁺ monocytes from SA

Clinical data of the groups of SA, AA, and NA investigated in our study are summarized in Table 1. Together, atopic individuals could be clearly distinguished from nonatopic individuals by their elevated serum IgE levels, their sensitizations to common environmental allergens and their stronger family background for atopy.

Of all Fc receptors tested, only the expression of the high-affinity receptor for IgE and FcεRI was found to differ significantly on CD14⁺ monocytes (Table 2). Both atopic groups showed enhanced expression of FcεRI compared with nonatopic individuals. The staining intensity of costimulatory molecules was not significantly different between the groups. However, CD40 displayed a tendency toward higher expression on CD14⁺ monocytes from SA than from AA and NA. When comparing the

expression intensities of the CD11 integrins, a marked difference was found for the expression of CD11c. The expression intensity of CD11c was significantly enhanced on CD14⁺ monocytes from individuals of the SA group compared with individuals of the AA and NA groups.

As the expression of costimulatory molecules on APC critically determines the outcome of T-cell activation and FcεRI has a likely role in the initiation of allergy, FcεRI⁺CD14⁺ monocytes of all three groups were analyzed for the expression of the T-cell costimulatory molecules CD40, CD80, and CD86 (Table 3). The results show that the intensity of the investigated costimulatory molecules on FcεRI⁺CD14⁺ monocytes did not differ significantly between the groups. Interestingly, there was a tendency toward a higher expression of CD86 on FcεRI⁺CD14⁺ monocytes from individuals of the AA group.

In summary, CD11c showed a significantly enhanced intensity on CD14⁺ monocytes from individuals presenting with the clinical manifestation of an allergic disease compared with asymptomatic atopic individuals and therefore suggests pathophysiological relevance for allergic inflammation.

Enhanced expression of FcεRI on CD14^{high}CD16^{dim} monocytes from AA

Monocyte subpopulations can be defined by two-color immunofluorescence analysis with antibodies against CD14 and CD16 (12). The majority of monocytes are CD16 negative and show strong CD14 staining (CD14^{high}CD16^{neg}). A second population with low levels of CD14 and high CD16 expression can be distinguished (CD14^{dim}CD16^{high}). In addition, a population of monocytes with an intermediate phenotype between these two main populations is present (Fig. 1A) (13). These cells show strong CD14 expression and low levels of CD16 (CD14^{high}CD16^{dim}). Three-color immunofluorescence analysis was performed for donor cells that had been established to express FcεRI on their monocytes (see Material and methods) to investigate FcεRI expression on these subpopulations. In donors of all groups the intensity of FcεRI was allocated predominantly to the 'classical' CD14^{high}CD16^{neg} monocytes (Fig. 1B).

Table 1. Clinical characteristics of symptomatic atopic (SA), asymptomatic atopic (AA) and nonatopic (NA) donors

	SA ($n = 10$)	AA ($n = 11$)	NA ($n = 21$)
Age (years)	33.4 (19–45)	29.7 (21–46)	31.1 (23–51)
Total IgE (kU/l)	302 (±261)	491 (±718)	22 (±30)
Atopic disease in early childhood (%)	70	18.2	0
Gender (female/male)	7/3	7/4	15/6
Positive family history of atopy (%)	60	45.5	23.8
Single/double sensitization to environmental allergens (%)	20	36.4	0
Multiple sensitizations (≥3) to environmental allergens (%)	80	63.6	0

Data are mean ± SD.

Table 2. Phenotypic characteristics of CD14⁺ monocytes from symptomatic atopic (SA), asymptomatic atopic (AA) and nonatopic (NA) donors. Relative fluorescence intensity (rFI) of cells $\pm$ SD

				P-value		
CD14 ⁺ monocytes	SA (n = 10)	AA (n = 11)	NA (n = 21)	SA/AA	SA/NA	AA/NA
Immunoglobulin receptors						
FcεRI	8.5 ± 6.8	7.9 ± 6.6	2.4 ± 2.1	0.833	0.007*	0.024*
FcεRII	2.0 ± 1.3	1.2 ± 0.8	1.2 ± 0.7	0.098	0.113	0.796
FcγRI	28.1 ± 7	29.4 ± 4	24.1 ± 8	0.398	0.220	0.065
FcγRII	5.6 ± 3.4	9.5 ± 6.5	8.5 ± 6.3	0.231	0.321	0.827
FcγRIII	11.2 ± 12.6	8.2 ± 5.4	8.2 ± 6.3	0.944	0.933	0.796
Costimulatory molecules						
CD40	7.1 ± 3.8	4.9 ± 2.1	4.9 ± 2.3	0.159	0.104	0.905
CD80	0.7 ± 0.2	0.6 ± 0.3	0.6 ± 0.3	0.647	0.485	0.984
CD86	3.8 ± 4.5	3.5 ± 2.5	3.9 ± 2.2	0.698	0.220	0.620
HLA-DR	10.1 ± 4.7	14.1 ± 11.3	14.8 ± 10.1	0.573	0.254	0.692
HLA-ABC	64.05 ± 28.9	95.3 ± 57.1	79.2 ± 39.4	0.360	0.353	0.565
CD11 integrins						
CD11a	42.9 ± 14	49.5 ± 14.9	58.9 ± 49.5	0.398	0.331	0.984
CD11b	185.4 ± 51.9	182.4 ± 46.3	161.3 ± 50.8	0.888	0.220	0.372
CD11c	78.9 ± 19.4	61.1 ± 9.8	55.8 ± 17.3	0.003*	0.001*	0.372
Other phenotypic marker						
CD123	3.9 ± 1.5	3.7 ± 0.7	4.5 ± 5.7	0.833	0.554	0.451

* Statistically significant.

Table 3. Intensity of costimulatory molecule expression on FcεRI⁺CD14⁺ monocytes from symptomatic atopic (SA), asymptomatic atopic (AA) and nonatopic (NA) donors. Relative fluorescence intensity (rFI) of FcεRI⁺CD14⁺ cells $\pm$ SD

				<i>P</i> -value		
FcεRI ⁺ CD14 ⁺ monocytes	SA (<i>n</i> = 8)	AA (<i>n</i> = 10)	NA (<i>n</i> = 7)	SA/AA	SA/NA	AA/NA
Costimulatory molecules						
CD40	6.4 ± 3.2	7.5 ± 3.4	7.6 ± 5.4	0.594	0.728	1.0
CD80	0.2 ± 1.0	0.03 ± 0.5	0.15 ± 0.3	0.859	0.487	0.769
CD86	13.6 ± 5.6	17.5 ± 7.7	13.0 ± 4.0	0.398	0.220	0.065

Interestingly, in individuals from the AA group, in contrast to donors of the SA and NA groups, FcεRI intensity was significantly enhanced on the intermediate type of CD14^{high}CD16^{dim} monocytes (Fig. 1C). On CD14^{dim}CD16^{high} monocytes from donors of all groups FcεRI intensity was low.

Together, monocyte subpopulations from atopic individuals without signs of allergic disease (AA) show phenotypic differences compared with symptomatic atopic and nonatopic individuals by enhanced expression of FcεRI on CD14^{high}CD16^{dim} monocytes.

Discussion

Monocytes are the putative precursors for myeloid DCs and macrophages, essential contributors to an allergic reaction (14). Understanding their role and functional properties *in vivo* is of major importance. The rationale of this study was to define factors that correlate with the manifestation of allergic inflammation independently of intrinsic properties related to atopy. Although reports have shown phenotypic differences between monocytes

from atopic and nonatopic individuals (15), this is the first study to delineate such differences between symptomatic and asymptomatic atopic donors.

In this study we identified the integrin CD11c to be expressed with a significantly higher intensity on the CD14⁺ monocyte surface of atopic individuals with AR in comparison with atopic individuals without allergic disease and nonatopic individuals. As the intensity of a defined surface antigen can determine the signal quality of a stimulus these results may be of functional relevance for the progression or prevention of allergic inflammation (16).

Monocytic differentiation is associated with the induction of CD11c/CD18 gene expression and thus, expression of CD11c is coupled to progression along the lineage of myeloid differentiation (17). The resulting CD11c/CD18 receptor mediates firm adhesion to other cells, to the vascular endothelium, transendothelial migration, chemotaxis and phagocytosis (18). In mice it is the CD11c⁺CD11b⁺ subset of DCs that captures airborne antigens and activates specific T cells long after antigen exposure (19). This suggests that CD11c⁺ DCs are able to sustain chronic Th2 mucosal inflammation even in the

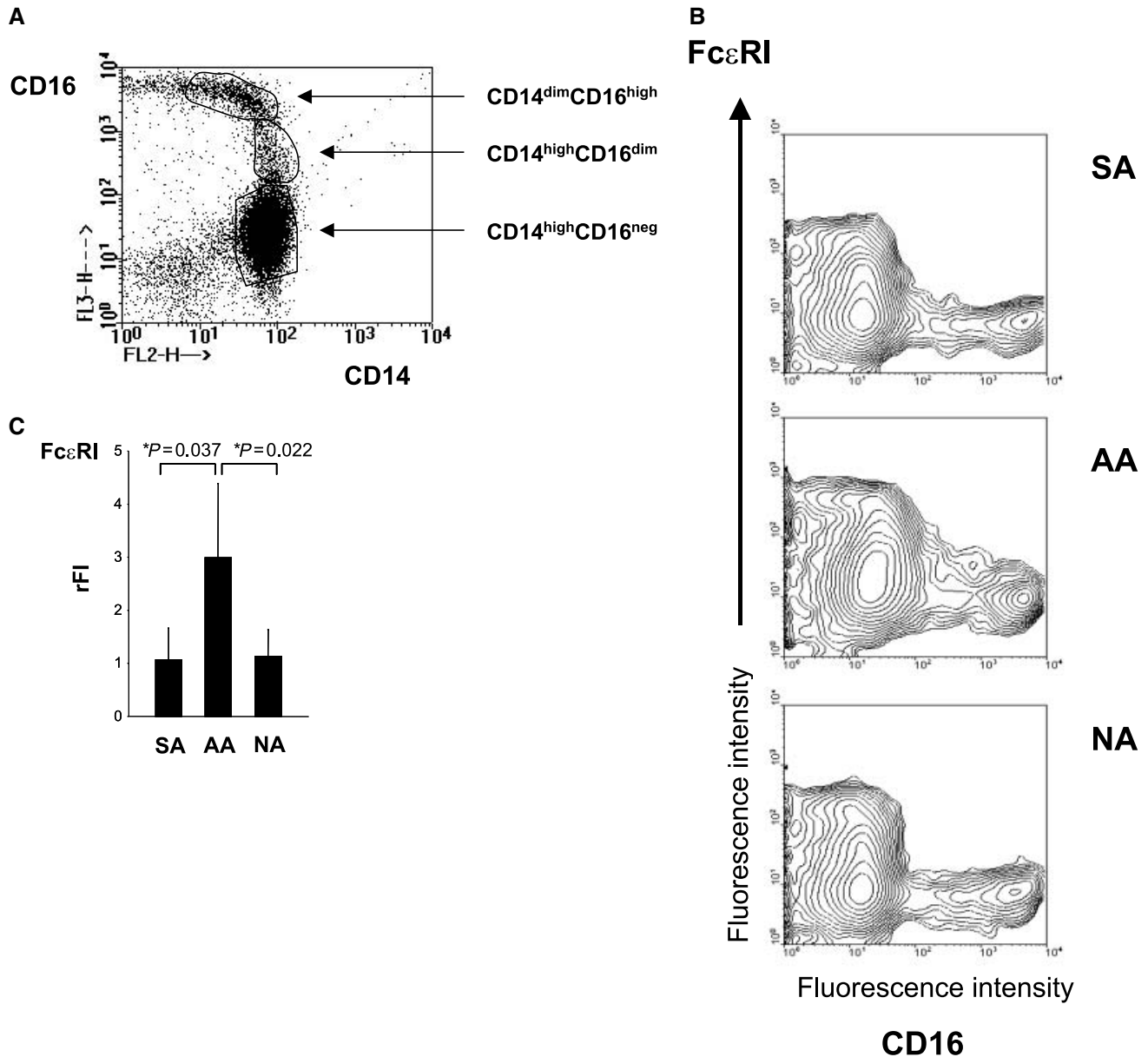


Figure 1. (A) Two-color immunofluorescence analysis of monocytes with mAbs directed against CD14 and CD16 antigens. The majority of monocytes are CD14^{high}CD16^{neg}. A population which is CD14^{dim}CD16^{high} can be clearly distinguished. In addition, an intermediate type of monocyte subset which is CD14^{high}CD16^{dim} can be found. (B) Three-color contour plot immunofluorescence analysis of monocytes with mAbs directed against CD14, CD16, and FcεRI. FcεRI intensity is highest on CD14^{high}CD16^{neg} cells from donors of all groups. In addition, in asymptomatic atopic individuals (AA), but not in symptomatic atopic (SA) and nonatopic (NA) individuals, FcεRI is enhanced on the CD14^{high}CD16^{dim} intermediate type of monocyte subset. Data are from one individual of each group and are representative of all SA ($n = 6$), AA ($n = 6$), and NA ($n = 7$) investigated. (C) FcεRI intensity on CD14^{high}CD16^{neg}, CD14^{high}CD16^{dim}, and CD14^{dim}CD16^{high} monocyte subsets of symptomatic atopic (SA; $n = 6$), asymptomatic atopic (AA; $n = 6$) and nonatopic (NA; $n = 7$) individuals. Data are expressed as mean $\pm$ SD.

absence of the relevant allergen. Our findings of an enhanced expression of CD11c on monocytes in SA might indicate that these cells have undergone differentiation into allergy-promoting effector cells. Inhibition of adhesion molecules such as CD11a in airway inflammation with monoclonal antibodies shows reduced allergen-

induced cellular inflammatory responses (20). Our study suggests that especially CD11c is involved in allergic pathophysiology and thus implicates CD11c as a good candidate for future therapeutic approaches. Higher expression of CD11c on monocytes may indicate that APCs are recruited more easily to sites of allergen entry

or remain there for prolonged periods, thereby promoting progression from atopy to allergy. Blocking of CD11c may thus help to slow down transmigration of monocytes into tissues, reduce the differentiation of monocytes into allergy-promoting CD11c⁺ DC and impair antigen presentation, all of which favor the manifestation of allergic disease.

As the interplay of costimulatory molecules on APCs with a T cell is important for T-cell activation or inactivation, we determined the expression intensity of several costimulatory molecules. However, none of the accessory surface molecules investigated on CD14⁺ and on FcεRI⁺CD14⁺ monocytes did show significantly enhanced or down-regulated surface intensity. Interestingly, on FcεRI⁺CD14⁺ monocytes from AA, CD86 intensity was almost significantly increased compared with monocytes from NA ($P = 0.065$). APCs can be flexibly programmed toward tolerance or immunity by ligation of specific surface receptors from complex environmental stimulation. For example, ligation of specific surface markers such as B7 (CD80, CD86) on normally immunogenic CD8⁺ murine splenic cells by soluble CTLA-4 is able to induce T-cell tolerance by induction of tryptophan catabolism. In contrast, CD40 ligation on tolerogenic CD8⁺ DC makes these cells capable of immunogenic presentation (21). The slight differences of costimulatory molecule expression on monocytes between donors of the SA and AA group detected in our study may indicate functional diversity of these molecules for T-cell activation/inhibition or be a reflection of ongoing immune regulation.

Ligation of FcεRI on monocytes mediates both immunostimulatory and immunoinhibitory functions (22, 23). Enhanced expression of FcεRI on the

CD14^{high}CD16^{dim} intermediate phenotype of monocyte subpopulations in asymptomatic individuals may indicate a special regulatory function of FcεRI on this monocyte subtype. Based on the concept that CD14^{dim}CD16^{high} monocytes derive from 'classical' monocytes by maturation into macrophages that includes down-regulation of CD14 and up-regulation of CD16 (24), it is conceivable that in AA, this intermediate monocyte subset derives from FcεRI⁺ CD14^{high} monocytes contrary to CD14^{low/neg} monocytes in SA. Detailed cytokine expression analysis as well as functional studies on monocyte subpopulations from these donors will be important to elaborate the significance of these findings.

In conclusion, our study implicates the integrin CD11c on monocytes as mediator for the manifestation of allergic disease. FcεRI expression on CD14^{high}CD16^{dim} monocytes from atopic individuals without clinical signs of allergies may have important regulatory functions to halt progression from atopy to allergy. The assignment of phenotypic differences between SA and AA donors to functional consequences will be important for future therapeutic interventions to prevent allergic inflammation.

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