

Research Paper

A rapid flow cytometric method for determining the cellular composition of bronchoalveolar lavage fluid cells in mouse models of asthma

Leonie S. van Rijt*, Harmjan Kuipers, Nanda Vos, Daniëlle Hijdra,
Henk C. Hoogsteden, Bart N. Lambrecht

*Department of Pulmonary and Critical Care Medicine, Erasmus University Medical Center (Room Ee22-57a),
Dr Molewaterplein 50, 3015 GE Rotterdam, The Netherlands*

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Abstract

Mouse models of allergic asthma are increasingly used to study the immunopathology of this complex disorder. The degree and type of airway inflammation is often studied by determination of differential cell counts on cytopspins of bronchoalveolar lavage fluid (BALF) cells stained with May–Grünwald Giemsa, in which the separation of eosinophils (eos) from neutrophils (neutro) and of monocytes (mono) from activated T cells can be quite problematic. In this study, we compared differential cell counts based on morphological criteria on May–Grünwald Giemsa stained cytopspins with a newly developed flow cytometric method. BAL fluid cells were identified based on forward and side scatter characteristics (FSC and SSC), autofluorescence of macrophages, and simultaneous one-step staining with antibodies for T cells (CD3-Cy-Chrome), B cells (B220-Cy-Chrome), eosinophils (CCR3-PE), and dendritic cells (DCs) (MHCII-FITC, CD11c-APC). The validity of this flow cytometric determination was tested by morphological analysis of flow-sorted cellular subsets. In an animal model of ovalbumin-induced asthma, this new method correlated very well with the differential counts based on cytopspins. Flow cytometric determination of the cellular composition of BAL fluid in mouse models of asthma is a rapid and easy method that can replace differential cell counts based on morphology. © 2004 Elsevier B.V. All rights reserved.

Keywords: Mouse; Lung; Asthma; Cell differentiation; BALF; Eosinophils; Flow cytometry

1. Introduction

Mouse models of allergic asthma are increasingly used to study the pathophysiology of this complex

disorder (Vargaftig, 1999; Lambrecht et al., 2000; Lloyd et al., 2001). In its simplest form, mice are sensitised to ovalbumin using either alum as an adjuvant or by using professional antigen presenting cells such as dendritic cells (DCs) as natural adjuvants (Lambrecht et al., 2000; van Rijt et al., 2002). Next, mice are challenged 10–14 days later with an aerosol of ovalbumin, and killed 24 h later. At this point, most models use bronchoalveolar lavage (BAL) to measure

Abbreviations: BALF, bronchoalveolar lavage fluid; DC, dendritic cell; Eo, eosinophil; FSC, forward scatter; SSC, side scatter.

* Corresponding author. Tel.: +31-10-408-7701; fax: +31-10-408-9453.

E-mail address: L.vanrijt@erasmusmc.nl (L.S. van Rijt).

the cellular composition of the lungs following allergen challenge. Bronchoalveolar lavage is also used as a clinical diagnostic tool in patients with a variety of lung disorders (Drent et al., 1994; Costabel and Guzman, 2001; Reynaud-Gaubert et al., 2002) and as a research tool in patients with asthma (Jarjour et al., 1998). In mouse models of asthma, the cellular composition of BAL fluid (BALF) is determined on cytopspins stained with either May–Grünwald Giemsa or hematoxylin–eosin. Using morphological criteria, it is at best possible to discriminate among macrophages/monocytes (mono), lymphocytes (lympho), neutrophils (neutro) and eosinophils (eos). Especially problematic in daily practice, however, is the ability to discriminate between eosinophils and neutrophils and between monocytes and activated T cells. Some authors have therefore used the endogenous cyanide-resistant peroxidase activity of eosinophils or the intracellular presence of major basic protein to enumerate these cells in BAL fluid (Ten et al., 1989; Boyce et al., 1995; Bell et al., 1996; Korsgren et al., 1997; Lee et al., 1997). In addition, morphological criteria are insufficient to discriminate between activated T and B cells, and between monocytes and alveolar DCs.

Flow cytometry and cell sorting have been used for several decades to identify and purify cells for different research goals. The development of a wide range of fluorescently labeled monoclonal antibodies has made it possible to analyse several cell types in large numbers of samples in a relatively short time period. Flow cytometry can combine ‘morphological’ features based on forward scatter/side scatter (FSC/SSC) channel intensity with expression of cell-specific surface markers using fluorescently labeled antibodies (Abs). It is therefore surprising that flow cytometry has hardly been used for the purpose of differential cell counting on BAL fluid samples in mice till now. By flow cytometric analysis, a multiplicity of cells can be analysed compared with the number of cells counted by an investigator (25,000 events vs. the traditional 200–400 counted cells in morphological analysis). In addition, staining and analysis of many samples simultaneously is much less time consuming compared with preparing, staining and counting cytopspins. Moreover, analysis by flow cytometry minimizes interobserver variation by rigorous determination of thresholds to determine the identity of a cell type.

Here we describe a flow cytometric method for differential cell counts of murine bronchoalveolar lavage fluid cells by staining with a combination of commercially available antibodies, MHCII, CCR3, CD3, B220 and CD11c. This combination of antibodies identifies T and B lymphocytes, eosinophils, neutrophils, dendritic cells and monocytes/macrophages in BALF.

2. Methods

2.1. Animals

Female BALB/c (H-2^d) mice (Harlan, Zeist, the Netherlands) were housed under specific pathogen-free conditions at the animal care facility at the Erasmus University Rotterdam. Mice were between 6 and 8 weeks old. The experimental procedure used in this study was approved by the Erasmus University Committee of Animal Experiments.

2.2. Generation of eosinophilic airway inflammation

On day 0, mice were actively immunized with OVA (grade V; Sigma, Missouri, USA) by intratracheal instillation of 1×10^6 OVA pulsed bone marrow DCs, and subsequently boosted at day 10 by an i.p. injection of 10 µg adsorbed to 1 mg of Al(OH)₃ (Sigma), as previously described (Lambrecht et al., 1998). Ten days after boosting, mice were challenged three times with a daily 30 min OVA or PBS aerosol [OVA grade III, 1% w/v in phosphate buffered saline (PBS); Sigma]. To induce a less severe airway inflammation in some mice, the OVA alum boost was omitted.

2.3. Flow cytometry

Twenty-four hours after the last aerosol, mice were sacrificed by avertin overdose followed by exsanguination. Bronchoalveolar lavage (BAL) was performed with 3×1 ml of Ca²⁺- and Mg²⁺-free PBS supplemented with 0.1 mM EDTA (Gibco). Red blood cells (RBCs) were lysed using ammonium chloride lysis buffer (van Rijt et al., 2002). Half of the cells were used for cytopspins and the remaining BALF cells were stained for 30 min in a refrigerator in a 96-well flexiplate (ICN, the Netherlands) with 40 µl FACS

buffer/well [PBS, 5% bovine serum albumin (BSA), 0.01% sodium azide] containing the final concentrations of the following monoclonals: MHCII-FITC (2.5 µg/ml; 2G9), allophycocyanin (APC)-labeled CD11c (2 µg/ml, HL3), Cychrome (CyChr)-labeled Abs against CD3 (10 µg/ml, 145-2C11) and B220 (2 µg/ml, RA3-6B2; PharMingen, Becton Dickinson, San Diego, CA, USA) and Phycoerythrin (PE)-labeled CCR3 (0.625 µg/ml; 83,101.111; R&D systems, Abingdon, UK). To prevent nonspecific binding to Fc receptors 2.4G2 blocking reagent (6 µg/ml) was added to the monoclonal antibody mix (See Table 1). Cells were sorted on a FACS DIVA and/or subsequently analysed on a FACS Calibur (BD biosystems) using CellQuest (Becton and Dickinson Immunocytometry Systems) and FlowJo software (Treestar, Costa Mesa, CA). Lymphocytes were identified as FSC^{lo}/SSC^{lo} and expressing CD3 or B220, B cells were distinguished from T cells by MHCII expression in the (B220/CD3)⁺ gate. Granulocytes were recognized as nonautofluorescent highly granular (SSC^{hi}) cells, and within this gate, eosinophils were defined as cells expressing the eotaxin receptor CCR3 (Grimaldi et al., 1999), inter-

mediate levels of CD11c (van Rijt et al., 2002), and very low to undetectable expression of MHCII (Van Rijt et al., 2003), B220 and CD3. Neutrophils had a similar scatter profile as eosinophils but lacked CCR3 expression (Grimaldi et al., 1999). Dendritic cells were identified as (CD3/B220)[−] and expressing high levels of MHCII and CD11c (Vermaelen et al., 2001; van Rijt et al., 2002). Alveolar macrophages cells were identified as large autofluorescent cells (Weyde et al., 1997).

2.4. Cytology

Cytospins were prepared from cells of the same BALF samples as used for flow cytometry. Slides were treated with 1% bovine serum albumin/PBS solution and 50,000 cells were spun onto the slides. Cytospins were dried overnight and then frozen at −80 °C until staining with May–Grünwald Giemsa and were characterized morphologically. In short, cytopins were stained by 3 min in May–Grünwald, 1 min in 1:1 May–Grünwald:milliQ, a short dip in milliQ, 20 min in 1:4 Giemsa:milliQ, two short dips in milliQ, dips in 0.01% acetic acid (glacial) until excess stain was removed, four dips in 95% ethanol, four dips in 100% ethanol and, finally, two dips in xylene. Stained cytopins were mounted in Entellan (See Table 1).

For histochemical detection of MHCII⁺ DCs in BALF, cytospin preparations of BALF cells were acetone fixed and blocked with 1% bovine serum albumin/PBS. MHCII expression was detected using a rat antimouse MHCII antibody (Ab) (M5.114), followed by goat antirat F(ab')₂ fragments conjugated to horseradish peroxidase (Serotec, Oxford, UK). The signal was developed using diaminobenzidine substrate for 10 min in TRIS buffered saline (Sigma). Cytospins were mounted in Entellan mounting medium (Merck, Darmstadt, Germany).

2.5. Purification of cell populations from the BALF

Cells were purified from BALF under sterile conditions using a FACS DIVA flow cytometric sorter (Becton Dickinson). BALF cells were stained as described above and different cell types were recognized based on scatter characteristics and expression of specific markers. Purity of the sort was determined at 84–99% by May–Grünwald Giemsa staining on cytopins of sorted cells. Four hundred cells were

Table 1

BALF cell differentiation	
Flow cytometry	Cytology
>100,000 cells/well flexiplate (up to 1×10^6)	Prewet slides with 1% BSA/PBS 50,000 cells/cytospin Dry overnight
Add 40 µl antibody mix/well: 2.4G2 (6 µg/ml)	3 min, May–Grünwald 1 min, 1:1 May– Grünwald:milliQ, short dip milliQ
MHCII FITC (2.5 µg/ml)	20 min, 1:4 Giemsa:milliQ
CCR3 PE (625 ng/ml)	Two short dips in milliQ, dips in 0.01% acetic acid
CD3 Cy-Chr (10 µg/ml)	Four dips in 95% ethanol
B220 Cy-Chr (2 µg/ml)	Four dips in 100% ethanol
CD11c APC (2 µg/ml)	Two dips in xylene
in FACS buffer (PBS, 5% BSA, 0.01% SoAz)	
Incubate 30 min at 4 °C in the dark	
Wash 150 µl FACS buffer (2 ×)	Dry 1 h
Spin down $400 \times g$ 3 min (2 ×)	
Resuspend in 150 µl FACS buffer	Mount in etellan
Acquire measurement at FACS	Dry overnight
Analyse with Flowjo	Count cells by morphological criteria

counted per cytospin. The determination of the validity of the sort was extended by admixing sorted eosinophils with BALF cells of naïve mice (which contained 0.73% eosinophils) at increasing concentrations (0%, 10%, 20%, 30%, ..., 100%). These mixed cell populations were measured by flow cytometry and the measured eosinophil percentages were compared with the admixed concentrations. A similar method was used by admixing bone marrow derived myeloid DCs (van Rijt et al., 2002) with BALF cells obtained from naïve mice.

2.6. Statistical analysis

Differential cell counts of BALF cells measured by flow cytometry were compared with cell counts obtained by cytology and using Spearman's rank correlation coefficient (SPSS 10.0 for Windows). The correlation was considered to be significant if $p < 0.05$.

3. Results

3.1. Identification of lymphocytes (T and B cells), eosinophils, neutrophils and mononuclear cells (macrophages and DCs) in BALF by flow cytometry

Mice were sensitised to OVA and subsequently challenged with three OVA aerosols from days 10 to 12. Twenty-four hours after the last aerosol challenge, the lung was lavaged. Bronchoalveolar lavage fluid cells were lysed with ammonium chloride to remove red blood cells. Cells were stained with a combination of antibodies including: MHCII-FITC, CCR3-PE, CD3-Cy-Chr, B220-Cy-Chr, CD11c-APC. To reduce nonspecific binding, cells were incubated with 2.4G2 blocking reagent.

Cells were analysed using six different parameters (Fig. 1). First, a plot of the FL3 channel detecting Cy-Chrome (Cy-Chr) and autofluorescent signal vs. cell size (FSC) was made. B220/CD3-Cy-Chr plotted against FSC was used to distinguish small B220/CD3⁺ lymphocytes from large autofluorescent macrophages and small CD3/B220[−] granulocytes (Fig. 1, first column of panels). In the second column of panels, cells were further identified using specific markers, following gating in the FL3/FSC plot. Small FSC^{lo} CD3/B220[−] granulocytes were further divided

in a MHCII/CCR3 plot into neutrophils or eosinophils upon expression of the eotaxin receptor CCR3-PE (Grimaldi et al., 1999). Within the small (FSC^{lo}CD3/B220^{hi}) lymphocyte gate, MHCII-FITC expression was used to discriminate T cells from MHCII⁺ B cells. FSC^{hi} large cells in BALF include autofluorescent macrophages and nonautofluorescent MHCII⁺/CD11c⁺ dendritic cells (see also Fig. 3C–F). The cell populations in BALF differed not only in size (FSC) but also in granularity (SSC) as shown in the third column of FSC/SSC plots gated on eosinophils, neutrophils, lymphocytes and mononuclear cells. Eosinophils are small cells that demonstrate considerable sideward light scattering properties, indicative of complex granules. Neutrophils, which are of identical size, have considerably less light scatter, illustrating the fact that the granular content is not so complex, but the scatter is still higher than with lymphocytes. Lymphocytes are small cells with very little light scattering ability. Mononuclear cells are large, medium to high light scattering cells, reflecting heterogeneity within this population.

To validate this classification based on specific markers and size/light scatter characteristics, all cell populations were flow cytometrically sorted and stained with May–Grünwald Giemsa (last column of panels). Eosinophils displayed a characteristic doughnut-shaped nucleus and had a pink cytoplasm in May–Grünwald Giemsa staining, indicative of eosinophilic granules. The sort of eosinophils was 93% pure (the remaining cells consisted of 6% mononuclear cells and 1% neutrophils). Neutrophils had a multilobed nucleus and the cytoplasm was only faintly stained with May–Grünwald Giemsa. The neutrophil sort was 84% pure (4% lympho, 6% mono, 6% eo). Lymphocytes had a rather large appearance and demonstrated a large round and partially lobed nucleus with a very high nuclear/cytoplasm ratio (99% pure sort with 1% eo). Mononuclear cells were large, sometimes veiled cells with a large cytoplasm (88% pure sort with 9% eo, 2% neutro, 1% lympho's).

3.2. Correlation of differential cell counts based on cytology and flow cytometry

Cytospins of BALF cells of OVA sensitised and challenged mice and control mice were stained with May–Grünwald Giemsa and differential cell counts

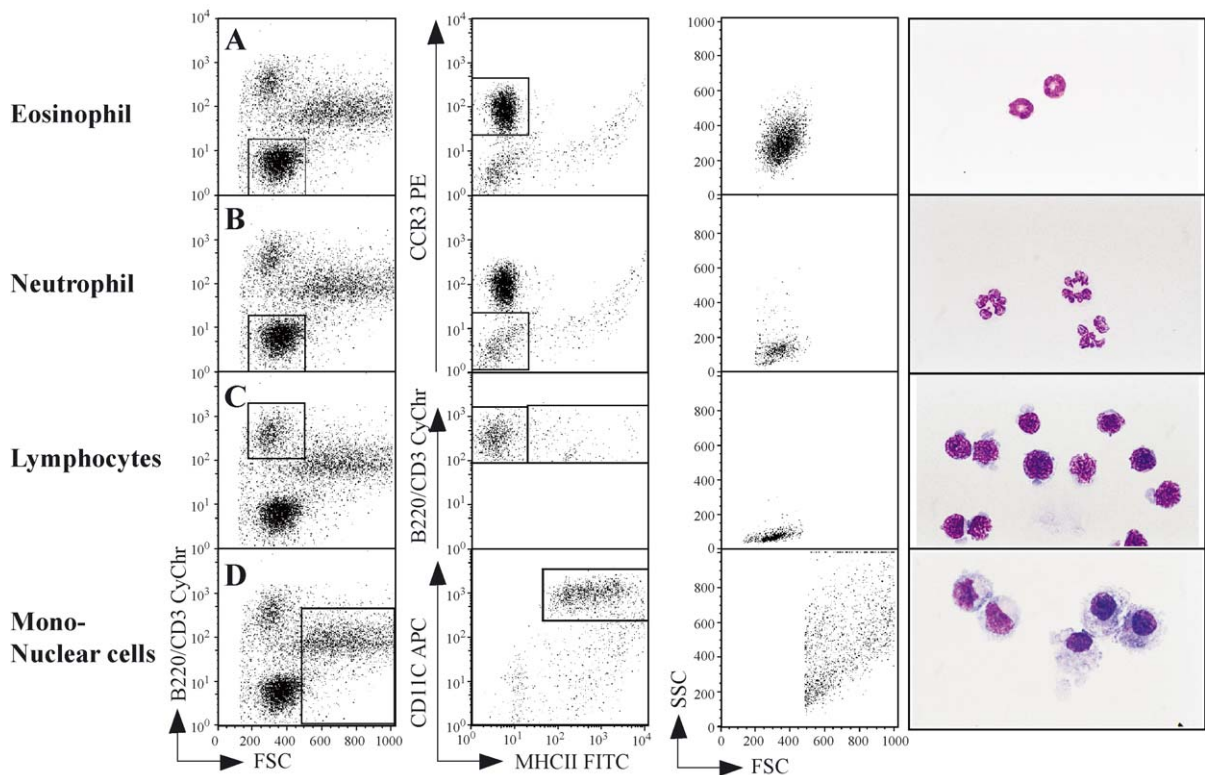


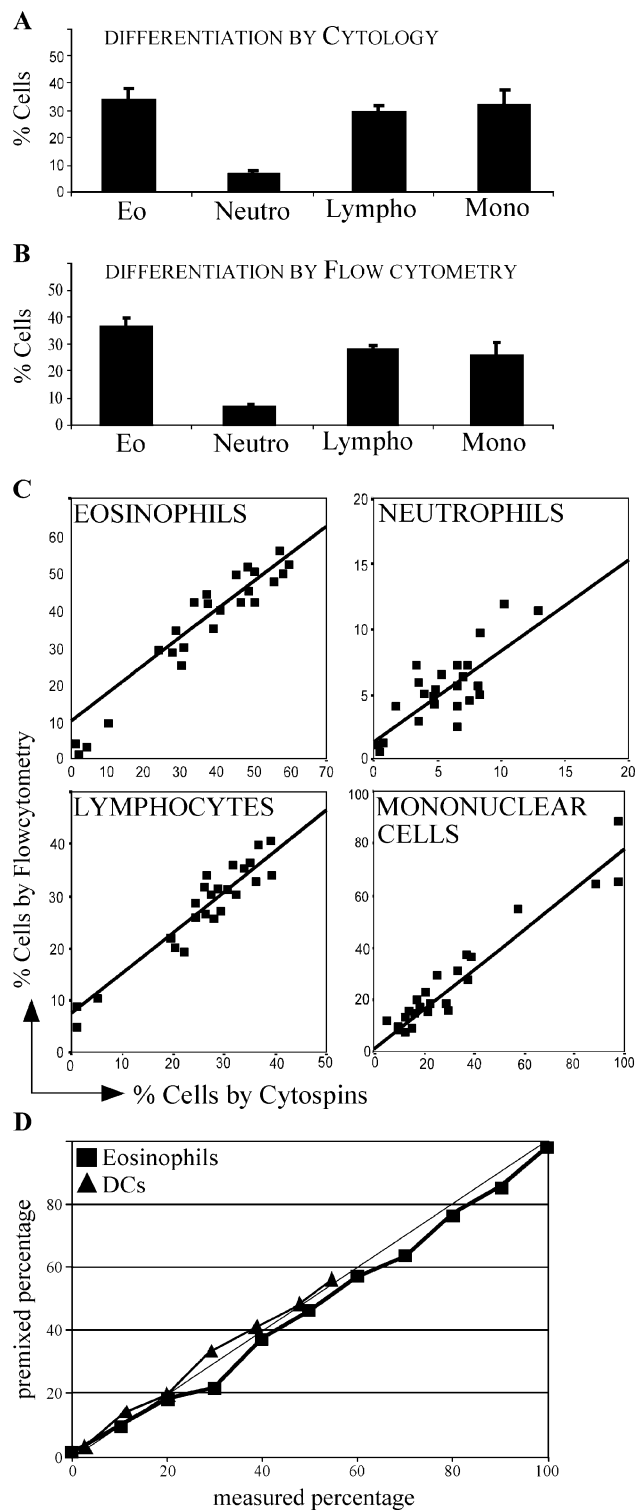
Fig. 1. Identification of major BALF cell types using flow cytometry. BALF cells were obtained from OVA sensitised and challenged mice. To determine the major BALF cell types, eosinophils (row A), neutrophils (row B), lymphocytes (row C) and mononuclear cells (row D) were stained with fluorescently labeled antibodies. First column of panels: CD3/B220 vs. forward scatter (FSC) plot, a gate was set on $(CD3/B220)^{-}$ granulocytes, $(CD3/B220)^{+}$ lymphocytes or mononuclear cells (high-autofluorescent macrophages and non-autofluorescent DCs). Second column: eosinophils were discriminated from neutrophils by the expression of the eotaxin receptor CCR3 within the granulocyte gate. B cells were distinguished from T cells by MHCII expression. Mononuclear cells had a high $MHCII^{+}/CD11c^{+}$ expression. Third column: 'morphological' flow cytometric parameters of the sorted cell types concerning size (FSC) and light scattering indicative of granularity (SSC). Fourth column: May–Grünwald Giemsa stained cytopspins of the sorted major BALF cell types, shown at $100\times$.

were performed by classification of 400 cells according to the morphological characteristics described above (Fig. 2A). BALF cells from the same mice were investigated for cellular composition by flow cytometry (Fig. 2B). The two differential cell count methods resulted in a similar cellular composition, and the two methods correlated very well. For each identified cell type, Spearman's rank correlation coefficient ρ was calculated. For eosinophils ($\rho=0.870$), neutrophils ($\rho=0.596$), lymphocytes ($\rho=0.789$) and mononuclear cells ($\rho=0.950$), the correlation coefficient was statistically significant (Fig. 2C, $p<0.05$). In addition to the validation of the method based on the morphological characteristics of the cell populations, we admixed sorted

BALF eosinophils with BALF cells of naïve mice and measured the cell mixtures on the FACS Calibur via the method described above. The premixed percentages of 0%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% and 100% eosinophils corresponded very well with the measured percentages (Fig. 2D). The same strategy was used by mixing bone marrow DCs with BALF of naïve mice.

3.3. Cellular composition of BALF in OVA sensitised/challenged mice compared to PBS sensitised/challenged mice

OVA aerosol challenge caused a strong eosinophilic inflammation of the lung of OVA sensitised mice



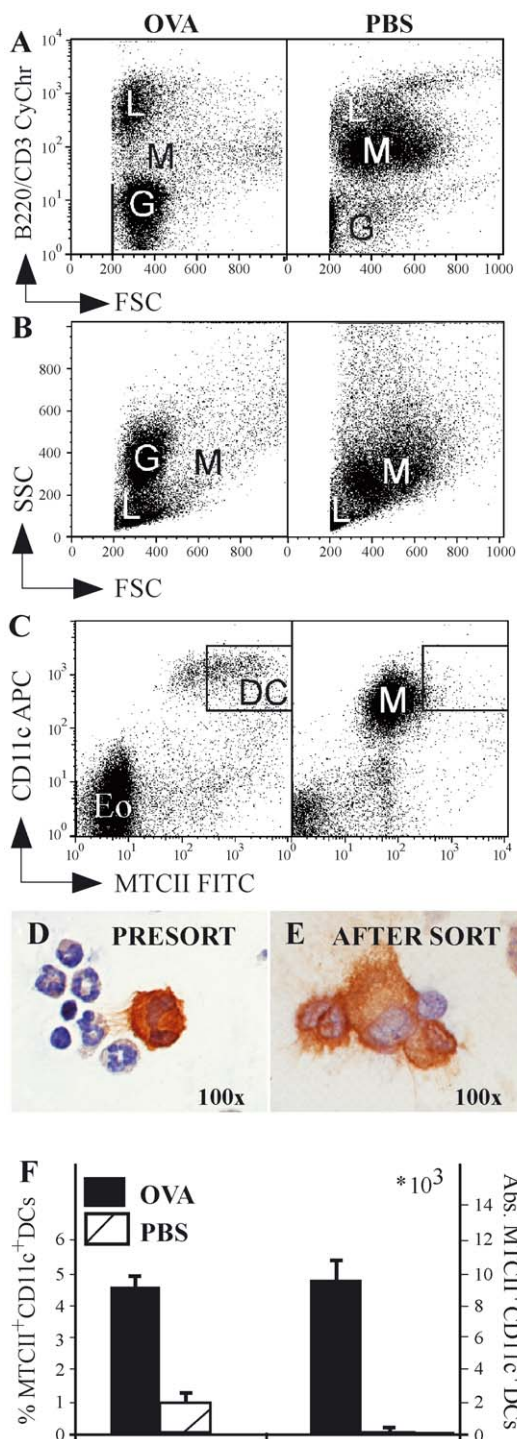
and this was reflected in the bronchoalveolar lavage fluid. On average, a total of $2.05 \times 10^6 \pm 1.95 \times 10^5$ cells were recovered. Performing the new flow cytometric method revealed that eosinophils accounted for 48.7%; macrophages for 13.8%; neutrophils for 9.5% and lymphocytes for 20.32% of the total cell count. In PBS sensitised/challenged animals, BALF cell composition consisted for the most part of alveolar macrophages (Fig. 3A–C) with some contaminating red blood cells and total cell counts were much lower ($8.98 \times 10^4 \pm 1.8 \times 10^4$). In the B220/CD3 against FSC plot (Fig. 3A) and FSC/SSC plot (Fig. 3B), the shift in cellular composition from mainly macrophages (M) during steady state towards granulocytes (G) and lymphocytes (L) during inflammation is clearly seen. Most of the granulocytes were CCR3⁺ eosinophils (see also Fig. 1A). Another distinct difference between PBS and OVA sensitised/challenged mice concerns the phenotype of mononuclear cells. In the steady-state condition, mononuclear cells are mainly CD11c^{int/hi}/MHCII^{int} macrophages, while in OVA sensitised and challenged mice large mononuclear cells demonstrated high CD11c and MHCII^{int/hi} expression, consistent with a DC phenotype. In order to demonstrate the presence of DCs in BAL fluid, MHCII staining on BALF cells was performed. This staining did indeed reveal (2.5% of total cells) large MHC class II strongly positive mononuclear cells with a dendritic morphology (Fig. 3D). The percentage determined by morphological analysis corresponded well with the 2.02% measured using flow cytometric analysis. Cells in the DC gate were sorted and MHCII staining was performed. The distinction between macrophages and DCs was made based on the higher level of MHCII expression and the smaller and/or lobed nucleus compared with macrophages. DC processes were observed after a period of 1 h at 37 °C in TCM containing 2% FCS (Fig. 3E). At least 84% of the sorted cells had a DC morphology (14.6% macrophages, 0.6% eos, 0.8% lympho). In OVA

sensitised mice, the number of these putative DCs was significantly enhanced, relatively as well as absolutely (Fig. 3F).

4. Discussion

Differential cell counting on BAL fluid cells is often used to ascertain the degree and type of inflammation induced by allergen challenge in mouse models of asthma (Lloyd et al., 2001). BAL fluid sampling is thought to reflect the degree of inflammation within and around the bronchial lumen and deeper alveolar compartments. Cell types are classically identified based on morphological criteria in May–Grünwald Giemsa staining using a combination of cell size, nuclear shape and staining of the cytoplasm. In daily practice, the differentiation of eosinophils from neutrophils can be quite problematic due to heterogeneity in cytoplasmic staining and the possibility that eosinophils can lose their cytoplasmic staining due to degranulation (Shinkai et al., 2002). Eosinophils are classically discriminated from neutrophils by the presence of eosinophilic cytoplasm, or by the presence of a ring-shaped nucleus. Another common problem in these differential cell counts of BAL fluid can be the discrimination between large activated lymphocytes and monocytes. Using a combination of cell size and granularity, autofluorescence, an antibody to the CCR3 receptor for eotaxin expressed on eosinophils, CD3 expressed on T cells, B220 expressed on B cells, MHCII expressed on B cells and DCs and CD11c expressed on DCs, we were able to identify six cell types. Eosinophils were found to be granular, non-autofluorescent, CCR3⁺ and CD11c^{dim} cells lacking expression of MHCII, B220 and CD3. Neutrophils, on the other hand, were also granular but lacked expression of CCR3 or CD11c. Lymphocytes were small nongranular, nonautofluorescent cells that expressed either CD3 (T cells) or B220 and MHCII (B cells).

Fig. 2. Correlation of differential cell counts of bronchoalveolar lavage fluid cells based on flow cytometry vs. histology. BALF cells were obtained from OVA sensitised and challenged mice ($n=20$) and PBS sensitised/OVA challenged control mice ($n=4$). The percentage of eosinophils, neutrophils, lymphocytes and macrophages in the BALF of mice were determined by counting 400 cells on May–Grünwald Giemsa stained cytopins using morphological criteria (A) or based on cytometric characteristics (B). Correlation between the percentage of each major cell type determined by FACS analysis and based on histology on BALF cells of OVA sensitised/OVA challenged mice and control mice (Spearman's correlation coefficient ρ was significant for all cell populations ($p<0.05$)) (C). In addition to the morphological validation of the method, sorted eosinophils (■) and cultured dendritic cells (▲) were admixed each with a pooled BALF sample of 10 naïve mice at increasing percentages and were subsequently measured by flow cytometry (D).



Dendritic cells were larger nonautofluorescent cells expressing high levels of CD11c and MHCII, while lacking expression of CCR3, CD3 or B220. Finally, macrophages were large autofluorescent cells expressing intermediate levels of CD11c. As far as we know, this is the first report of murine BALF cell differentiation using flow cytometry instead of cytological differentiation on cytopins. The validity of our staining reaction was ascertained by flow cytometrically sorting the various populations identified and looking at the morphology of the cells. The validation of the method was furthermore verified by flow cytometry via admixing sorted eosinophils (purity 98%, based on flow cytometric analysis of the sorted sample) or cultured dendritic cells with BALF cells of naïve mice at increasing concentrations. The admixed percentages corresponded very well to the measured percentages. We also compared the flow cytometric method with the histological method to determine cellular composition of BALF using the same samples. The resulting differential cell counts of BALF based on flow cytometric characteristics correlated very well with those based on the classical morphological criteria. In particular, the degree of BAL fluid eosinophilia, a primary endpoint in most studies on murine asthma, correlated extremely well across a broad range making this method very useful indeed.

Fig. 3. Flow cytometric characteristics and cellular composition of BALF cells in OVA sensitised/challenged mice compared with PBS sensitised/challenged mice. Cellular composition of BALF cells of OVA sensitised/challenged mice (left column) compared with PBS sensitised/challenged mice (right column). (A) Ungated BALF cells in a B220/CD3 vs. FSC plot. In PBS sensitised/challenged mice, mainly autofluorescent macrophages ('M') were recovered from the bronchoalveolar compartment. In OVA sensitised/challenged mice, granulocytes (mainly eosinophils, see Fig. 1) ('G') and lymphocytes ('L') were attracted to the site of inflammation. (B) Distinction of eosinophils, lymphocytes and macrophages based on the morphological flow cytometric parameters FSC and SSC. (C) CD11c vs. MHCII plot on ungated cells. In OVA sensitised/challenged mice, CD11c^{lo}/MHCII^{lo} eosinophils and CD11c^{hi}/MHCII^{hi} DCs are present. In PBS sensitised/challenged mice, macrophages have a CD11c^{int}/MHCII^{int} phenotype and CD11c^{hi}/MHCII^{hi} DCs are absent. (D) MHCII staining on unsorted pooled BALF cells of OVA sensitised/challenged mice. (E) MHCII staining on sorted CD11c^{hi} MHCII^{hi} cells showing large dendrites, typical of DCs. (F) The number and percentage of CD11c^{hi}/MHCII^{hi} DCs in BALF of OVA sensitised/challenged mice compared to PBS sensitised/challenged mice.

The use of specific antibodies made it possible to further characterize several distinct cell types not classically mentioned in manual counts of cytopspins. Within the B220/CD3-positive lymphocyte gate, formed by gating on small nongranular cells expressing CyChr-labeled CD3 and B220, expression of MHCII made it possible to distinguish B cells from T cells instead of gathering the two cell types as lymphocytes. In mice, in contrast to humans, activated T cells do not express MHC class II allowing differentiation of B cells based on this marker.

Mononuclear cell types are often lumped together in manually counted differential formulae. In the lung, these cells consist mainly of alveolar macrophages with a very high degree of autofluorescence. However, immunostaining for MHC class II revealed that numerous cells with long dendrites could be found in the airways of allergen challenged mice, most likely representing DCs (van Rijn et al., 2002; Vermaelen and Pauwels, 2003). By using the DC-specific marker CD11c in combination with high level expression of MHC class II on flow cytometry, we were able to show that DCs were also contained in the population of cells classically enumerated as mononuclear cells or monocytes/macrophages. Immunohistological staining of MHCII on sorted MHCII^{hi}CD11c^{hi} cells showed cells with, unsurprisingly, a high MHCII expression, a low nucleus/cytoplasm ratio and a smaller and/or lobed nucleus compared with the round and larger nucleus of macrophages, and numerous long dendrites. The percentage DCs in BALF of mice that were OVA sensitised and challenged, based on cytopspins (2.5%), corresponded well to the percentage found by flow cytometry on the same BALF cells (2.02%). In baseline conditions, mononuclear cells consisted mainly of autofluorescent CD11c^{hi} MHC-II^{int} alveolar macrophages. It is very hard to study the specific expression of fluorescent markers on alveolar macrophages that spontaneously emit light at all the detection channels of the flow cytometer, and therefore it cannot completely be excluded that part of the signal of CD11c and MHC class II is due to autofluorescence (Weyde et al., 1997). However, others have reported weak CD11c expression and MHC class II by 10–30% of alveolar macrophages (Sertl et al., 1986). As we challenged the lungs with allergen, the cellular composition of BALF shifted from mainly macrophages to recruited eosinophils and nonautofluorescent CD11c^{hi} MHC-II^{hi} cells.

It has been shown that macrophages can mature into dendritic cells by incubating macrophages overnight in the presence of GM-CSF (Palucka et al., 1998; Rezzani et al., 1999; Zou et al., 2000; Suzuki et al., 2001; Nikolic et al., 2003). We hypothesize therefore that these CD11c^{hi}/MHCII^{hi} cells are mature dendritic cells (Fig. 3C) that were either recently recruited from the bloodstream (van Rijn et al., 2002) or that developed from CD11c^{hi}/MHCII^{int} alveolar cells under the influence of locally produced cytokines. Additional experiments will have to demonstrate whether alveolar macrophages indeed give rise to DCs under the inflammatory conditions offered by eosinophilic airway inflammation.

Although the method described in this report is relatively simple, certain points should be addressed. First, alveolar macrophages display a good deal of autofluorescence, which can be measured at all detectors to various extents, and can significantly disturb measurements of cell-specific markers (Roederer, 2000). Therefore, care should be taken to exclude these cells from the granulocyte and lymphocyte gate. Second, degranulated eosinophils probably have a different light scattering phenotype compared to eosinophils that have not degranulated. After degranulation, eosinophils give a much lower side scatter signal due to the expulsion of complex granules (Shinkai et al., 2002). In our hands, all CCR3⁺ cells had a highly granular phenotype on FSC/SSC plots arguing against significant degranulation. Indeed, in several asthma models, the degranulation of eosinophils has been questioned (Denzler et al., 2001; Malm-Erfjelt et al., 2001). However, it could be that in other asthma models, the degranulation of eosinophils would give rise to CCR3⁺ cells of low side scatter profile.

Identification of major cell type populations by flow cytometry automatically leads to the possibility of isolating these cells by flow-sorting. In this way, contamination with other cell types can be specifically prevented. Others have used the characteristic of eosinophils to emit polarized scattered light to sort these cells from lavage fluid (MacKenzie et al., 2001). We have studied several BALF cell types identified on flow cytometric characteristics (van Rijn et al., 2002, 2003). Using the same criteria to isolate BAL fluid cells as described here, we were able to investigate the presumed antigen presenting capacity of BAL fluid eosinophils without contamination of other antigen

presenting cells, like B cells, macrophages and DCs. Using this method, we were able to sort large quantities of eosinophils at a very high purity (>98%). Other applications are the sorting of BAL fluid subsets (e.g., DCs or T cells) to perform subsequent RT-PCR of expressed genes (unpublished observations).

In conclusion, we have developed a new, rapid and simple method to determine the cellular composition of BALF cells in a murine model for asthma using flow cytometry. This method correlated very well with differential cell counts based on morphological characteristics and may prove to be a time-saving, investigator-independent and more detailed method for general use in experimental asthma research.

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