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Research Article

Isolation of myeloid and plasmacytoid dendritic cells from human bronchoalveolar lavage fluid

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Summary Studies of bronchoalveolar lavage fluid (BALF) dendritic cells (DC) have been hampered by the scarcity of DC and the lack of DC-specific surface markers. Four surface Ag have been recently described as specific markers for distinct subsets of DC and have been used for the isolation and characterization of fresh noncultured DC from lung resection specimens: BDCA-1 (CD1c) and BDCA-3 for myeloid DC type 1 and type 2, respectively, and BDCA-2 and BDCA-4 for plasmacytoid DC. The aim of this study was to develop a new method for the isolation of BALF DC, using immunomagnetic separation of BDCA+ cells. Mononuclear cells were obtained from BALF after Ficoll-Paque density gradient centrifugation. Monocytes, T cells and B cells were magnetically labelled and depleted. The unlabelled cell fraction was incubated with BDCA-1, BDCA-3 and BDCA-4 beads and the total BDCA+ DC were retained. The ability of isolated DC to induce T-cell responses was evaluated by coculturing the isolated DC with immunomagnetically sorted naive T cells. The above procedure resulted in a population of viable DC that showed a strong capacity in induce T-cell responses. Functionally intact human BALF myeloid DC types 1 and 2 as well as plasmacytoid DC can be easily obtained by immunomagnetic isolation. Considering that bronchoalveolar lavage is a minimally invasive procedure, these cells are optimal candidates with which to elucidate the properties and capabilities of pulmonary DC.

Key words: BDCA, bronchoalveolar lavage fluid, CD1a, dendritic cell, Langerhans cell.

Introduction

Among APC, dendritic cells (DC) are unique in being able to prime innate and adaptive immune responses and efficiently stimulate memory responses. Evidence is also mounting that DC play a pivotal role in directing type 1 or type 2 effector T-cell responses and in inducing T-cell tolerance.[1,2] DC are particularly localized to sites of intense exposure to antigenic stimuli (i.e. the skin and mucosal surfaces), such as those of the lung. Pulmonary DC are present within the airway epithelium, submucosa and alveolar surface and within the lung parenchyma.[3] Under homeostatic conditions, they express an immature phenotype, but after Ag exposure and in the context of danger signals such as inflammation, pathogen invasion or tissue damage, pulmonary DC undergo maturation, which ensures expression of molecules relevant for T-cell stimulation and migration to the secondary lymphoid organs, where naive T cells reside in large numbers.[3]

DC are being increasingly studied for their possible involvement in the pathogenesis of several pulmonary diseases, and there are now enough data that support a central role for DC in respiratory infections, lung cancer, asthma and smoke-related diseases.[4–9] DC are commonly generated by *in vitro* differentiation of monocytes with exogenous cytokines.[10] However, monocyte-derived DC differ in their molecular phenotype and function from tissue DC.[10] DC represent only 1–2% of circulating PBMC and approximately 0.5% of bronchoalveolar lavage fluid (BALF) cells. Isolation of pulmonary DC has been hampered by their scarcity and by the lack of DC-specific markers.[11–13] Early methods of human pulmonary DC isolation used enzyme-digested tissue and overnight incubation for enrichment of transiently adherent mononuclear cells, which could induce phenotypical and functional alterations in the cells.[14]

Sorting experiments on BALF and lung digests have used low autofluorescence as a criterion to isolate DC.[13–15] Recently, Demedts *et al.* suggested a new protocol for the isolation of functionally intact human lung DC from surgical resection specimens in which three novel specific markers for distinct subsets of DC are used to label and sort low-autofluorescent cells: BDCA-1 (CD1c) and BDCA-3 for myeloid DC (MDC) type 1 and type 2, respectively, and BDCA-2 for plasmacytoid DC (PDC).[16,17] However, analysis of DC from lung surgical specimens is limited to subjects undergoing lobectomy or pneumectomy for medical reasons, mostly for lung cancer. Moreover, tissue digestion might cause damage to cell functions and to cell surface structures.

Bronchoalveolar lavage (BAL) is a minimally invasive procedure that permits the recovery of airway–alveolar space cells for research purposes from human BALF.[18] Human BALF contains small numbers of MDC and PDC, and van Haarst *et al.* have succeeded in sorting a population of low-autofluorescent CD1a$^+$ cells, also designated as Langerhans cells.[13] Isolation

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of the other three major lung DC subpopulations (MDC types 1 and 2 and PDC) from human BALF has not been reported yet.

Magnetic activated cell sorting protocols have been used for the effective isolation of functionally intact human epidermal Langerhans cells.[19] Based on immunomagnetic separation of BDCA-labelled cells, we have developed a new protocol for the isolation of human BALF MDC types 1 and 2 and PDC, for the first time. The isolation of functionally intact MDC and PDC from human BALF is expected to be crucial for the elucidation of the phenotypic and functional properties of human lung DC.

Materials and methods

Bronchoalveolar lavage

BAL was carried out on 15 subjects undergoing fibre-optic bronchoscopy for various medical purposes. The protocol was approved by the Ethics Committee of the University Hospital of Heraklion, and all subjects gave their written informed consent. The protocol conformed to the provisions of the Declaration of Helsinki (as revised in Edinburgh 2000), available at http://www.wma.net/e/policy/be.htm.

BAL was carried out with a flexible bronchoscope placed on the right middle lobe of the lung with the tip in wedge position. Four aliquots of 50 mL isotonic saline were instilled, recovered by gentle aspiration and collected in siliconized bottles, to prevent cell adherence. BALF volume ranged from 25 to 120 mL.

Isolation of DC

BALF was kept at 4°C and processed within 1 h. BALF was filtered through a 70 μm gauze (352350; BD Pharmingen, San Diego, CA, USA). BALF mononuclear cells (BALF MC) were obtained by centrifugation at 1000 *g* for 30 min at 4°C over Ficoll–Paque density gradients (10771; Sigma Diagnostics, St Louis, MO, USA). BALF MC were first depleted of T cells, using anti-CD3 beads according to the manufacturer's instructions (130-050-101; Miltenyi Biotec, Bergisch Gladbach, Germany). Then, a commercially available DC isolation kit, which has been developed for the isolation of MDC types 1 and 2 and PDC from PBMC, was used according to the manufacturer's instructions (130-091-379; Miltenyi Biotec). Although PDC express both BDCA-2 and BDCA-4, we used BDCA-4 microbeads for the magnetic labelling because BDCA-2 inhibits IFN-α production by DC.[20] In brief, cells were incubated with anti-CD14 beads, anti-CD19 beads and a biotin-conjugated antibody against BDCA-1 (130-091-379; Miltenyi Biotec). Then, the magnetically labelled monocytes ($CD14^+$ cells) and B cells ($CD19^+$ cells) were depleted. Subsequently, cells were incubated with anti-BDCA-3 beads, anti-BDCA-4 beads and anti-biotin beads for magnetic labelling of biotin-labelled BDCA-1+ DC (130-091-379; Miltenyi Biotec). On subsequent magnetic separation, the magnetically labelled DC were retained. To achieve higher purity, the positively selected fraction was separated over a second column. The viability of the isolated cell population was more than 70% (judged by Trypan blue staining).

Evaluation of DC purity

The purity of the isolated BALF DC was evaluated by flow cytometry. The mAbs used are listed in Table 1. For the identification of MDC and PDC, two different strategies were followed. The first strategy was based on the identification of BDCA antigens. MDC type 1 were identified on the basis of staining with BDCA-1 (CD1c), MDC type 2 were identified on the basis of staining with BDCA-3 and PDC were identified on the basis of staining with BDCA-2. B cells, which also express BDCA-1, and monocytes, which express BDCA-3 at low levels, were excluded from the analysis on the basis of staining with CD19 and CD14, respectively.

The second strategy was based on the presence of CD85k (ILT3) and CD33 or CD123. CD85k is selectively expressed on monocytes, macrophages and DC.[21] CD33 is strongly expressed on MDC of both types, whereas CD123 is strongly expressed on PDC.[16] MDC of types 1 and 2 were identified on the basis of double staining with CD33 and CD85k, whereas PDC were identified on the basis of double staining with CD123 and CD85k. Monocytes and macrophages, which also express CD85k, were excluded from the analysis on the basis of staining with CD14 and CD16.

For further evaluation of the isolated cell population, the following antibodies were used: the anti-HLA-DR antibody, which is expressed by most DC and B cells, and by some T cells and macrophages; the anti-CD45 pan-leucocyte marker; the anti-CD3 T-cell-specific marker; the anti-CD16 antibody, which is specific for NK cells, granulocytes and macrophages; the anti-CD56 NK-cell-specific marker; the anti-CD15 granulocyte-specific marker; the anti-CD14 monocyte/macrophage-specific marker; and the anti-CD19 B-cell marker. The specific mAb clones and fluorescence colours are presented in Table 1.

Isolation of peripheral blood naive T cells

Anticoagulated peripheral blood was diluted 1:1 with RPMI-1640 medium (21875-034; Gibco Invitrogen, Carlsbad, CA, USA). PBMC were obtained by centrifugation at 1000 *g* for 30 min at room temperature over Ficoll–Paque density gradients (Sigma Diagnostics). Untouched T cells were isolated from PBMC, using a commercially available kit (130-091-156; Miltenyi Biotec). In brief, non-T cells (i.e. B cells, NK cells, DC, monocytes, granulocytes and erythroid cells) were indirectly magnetically labelled with a cocktail of biotin-conjugated mAbs directed against CD14, CD16, CD19, CD36, CD56, CD123 and Glycophorin and anti-biotin mAbs conjugated to microbeads. Isolation of pure T cells was achieved by depletion of magnetically labelled non-T cells. Naive T cells were separated after incubation with anti-CD45RA beads and positive selection of $CD45RA^+$ T cells (130-045-901; Miltenyi Biotec). The viability of naive T cells was more than 96% (judged by Trypan blue staining). The purity of naive T cells was evaluated by flow cytometry using anti-CD3 and anti-CD45RA antibodies and was always >98%. The mAbs used are listed in Table 1.

DC–T-cell coculture

Naive T cells were cocultured with BALF DC from the same subject at a 4:1 ratio in 200 μL Yssel's medium (400-103; Gemini Bioproducts, Woodland, CA, USA) containing 10% fetal bovine serum (FBS [F9665; Sigma]) in 96-well round-bottom culture plates for 4 days. T cells were also cultured with anti-CD3 (5 μg/mL [555329; BD Pharmingen]) and anti-CD28 (1 μg/mL [555725; BD Pharmingen]) antibodies coated on culture plates for 4 days at 37°C under a 5% CO_2 fully humidified atmosphere.

Analysis of intracellular cytokine production

After 4 days of priming, T cells were restimulated with soluble anti-CD3 (5 μg/mL) and anti-CD28 (1 μg/mL) for 5 h under the same culture conditions. Brefeldin A (1 μg/mL [B7651; Sigma]) was added to the cultures for 2 h before the staining to prevent cytokine secretion. Harvested cells were washed, incubated with mouse serum (M5905; Sigma), fixed with 2% formaldehyde (017 5483104B; Merck, NJ, USA) and permeabilized with PBS (18912-014; Gibco Invitrogen)

Table 1 mAbs used for flow cytometry

Specificity	Clone	Isotype	Conjugate	Source
mAbs used for evaluation of DC purity				
BDCA-1	AD5-8E7	$mIgG_{2a}$	PE	Miltenyi Biotec, Bergisch Gladbach, Germany
BDCA-2	AC144	$mIgG_1$	FITC	Miltenyi Biotec
BDCA-3	AD5-14412	$mIgG_1$	PE	Miltenyi Biotec
CD3	UCTHT1	$mIgG_1$	FITC	Beckman Coulter Immunotech, Marseille, France
CD14	TÜK4	$mIgG_{2a}$	PE-Cy5	Miltenyi Biotec
CD14	RMO52	$mIgG_{2a}$	FITC	Beckman Coulter Immunotech
CD15	80H5	mIgM	PE	Beckman Coulter Immunotech
CD16	3G8	$mIgG_1$	FITC	Beckman Coulter Immunotech
CD19	LT19	$mIgG_1$	PE-Cy5	Miltenyi Biotec
CD19	J4.119	$mIgG_1$	PE-Cy5	Beckman Coulter Immunotech
CD33	D3HL60	$mIgG_1$	PE-Cy5	Beckman Coulter Immunotech
CD45	J.33	$mIgG_1$	PE-Cy5	Beckman Coulter Immunotech
CD56	N901	$mIgG_1$	PE	Beckman Coulter Immunotech
CD85k (ILT3)	ZM3.8	$mIgG_1$	PE	Beckman Coulter Immunotech
CD123	107D2	$mIgG_1$	PE-Cy5	Beckman Coulter Immunotech
HLA-DR	Immu-357	$mIgG_1$	PE	Beckman Coulter Immunotech
mAbs used for evaluation of naive T-cell purity				
CD3	UCTHT1	$mIgG_1$	FITC	Beckman Coulter Immunotech
CD45RA	ALB11	$mIgG_1$	PE	Beckman Coulter Immunotech
mAbs used for intracellular cytokine detection				
IFN-γ	45.15	$mIgG_1$	PE	Beckman Coulter Immunotech
IL-4	4D9	$mIgG_1$	PE	Beckman Coulter Immunotech
IL-10	25209.1	$mIgG_1$	PE	BD Pharmingen, San Diego, CA, USA
Controls				
Control 1	679.1Mc7	$mIgG_1$	FITC	Beckman Coulter Immunotech
Control 2	679.1Mc7	$mIgG_1$	PE	Beckman Coulter Immunotech
Control 3	679.1Mc7	$mIgG_1$	PE-Cy5	Beckman Coulter Immunotech
Control 4	543.10	$mIgG_{2a}$	FITC	Miltenyi Biotec
Control 5	GC323	mIgM	PE	Beckman Coulter Immunotech

DC, dendritic cell; PE, phycoerythrin; PE-Cy5, phycoerythrin-Cyanine 5.

supplemented with 2% FBS (F9665; Sigma), 0.1% NaN_3 (58032-25G; Sigma) and 0.5% saponin (S4521; Sigma). Cells were stained with fluorochrome-labelled anti-IFN-γ or anti-IL-4 or anti-IL-10. The mAbs used are listed in Table 1.

Flow cytometry

An ELITE COULTER cytometer (Beckman Coulter Immunotech, Fullerton, CA, USA) was used for three-colour flow cytometry. Data for 5×10^3 to 2×10^5 cells/sample were acquired in list mode and analysed using Epics Elite (Beckman Coulter Immunotech). All antibodies used were conjugated to phycoerythrin (PE), FITC or phycoerythrin-Cyanine 5 (PE-Cy5). Isotypic control antibodies were used as negative controls.

Results

DC purity

We were able to isolate BALF DC from 13 out of 15 (87%) patients included in the study: ten patients with lung cancer, two healthy smokers with haemoptysis and one patient with interstitial lung disease. In two patients, the procedure yielded a very small cell population, insufficient for further evaluation with flow cytometry.

The purity of BALF DC was evaluated by flow cytometry. First, cells were gated based on their side scatter and forward scatter properties. Dead cells and debris were excluded (Fig. 1A). Then, B cells ($CD19^+$ cells) and monocytes ($CD14^+$ cells) were excluded from the analysis (Fig. 1B) and MDC types 1 and 2 were identified in the negative fraction as BDCA-1+ and BDCA-3+ cells, respectively, whereas PDC were identified by staining with anti-BDCA-2 (Fig. 1C–H). Because BDCA-3 is also expressed at much lower levels on monocytes, granulocytes, MDC type 1 and PDC, only events with a high BDCA-3 fluorescent signal were included in the analysis (Fig. 1H). mAbs directed against $CD19^+$ and $CD14^+$ cells, along with mAbs that matched the isotype of the anti-BDCA antibodies, but had irrelevant specificities, were used as controls (Table 1). Patient A represented in Figure 1 shows higher numbers of MDC compared with PDC. Figure 2 shows results from an independent experiment in a different patient (patient B) with a higher number of PDC compared with MDC. Results were expressed as the percentage of gated DC among total isolated cells (cells gated in scatter modes) and as absolute numbers (Fig. 3). The purity of DC was assessed by adding the percentage of each DC subpopulation (Fig. 3A). Absolute DC numbers were calculated by multiplying DC percentages by the absolute number of total isolated cells (Fig. 3B).

In a different gating strategy, MDC (both types 1 and 2) were identified as double-positive CD85k and CD33 cells, whereas PDC were identified as double-positive CD85k and CD123 cells, in the CD14- and CD16-negative cell fraction. Results

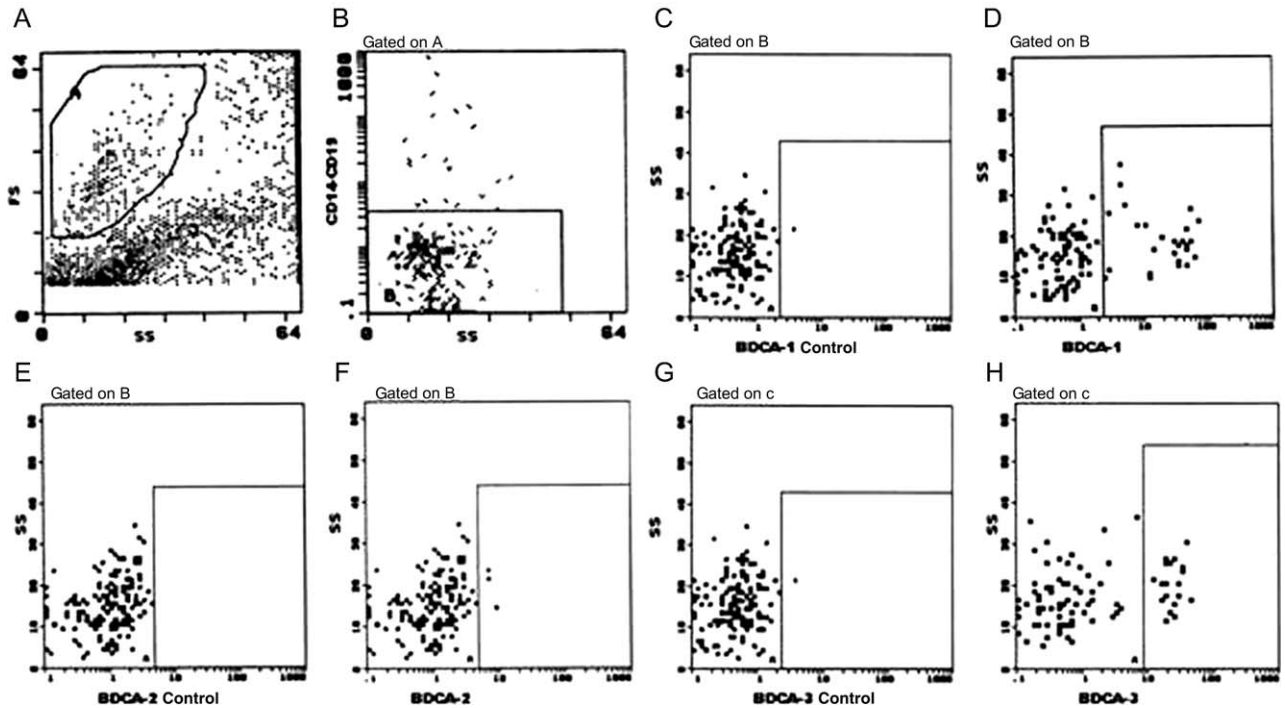

Figure 1 Detection of MDC1, MDC2 and PDC in isolated bronchoalveolar lavage fluid cells after non-DC depletion and BDCA+ cell selection in patient A. Panel A shows gating in forward scatter (FS) and side scatter (SS) modes to exclude dead cells and debris. Panel B shows gating to exclude B cells ($CD19^+$) and monocytes ($CD14^+$). Panel C shows gating of the BDCA-1 control sample. Panel D shows gating to include BDCA-1+ cells (MDC1). Panel E shows gating of the BDCA-2 control sample. Panel F shows gating to include BDCA-2+ cells (PDC). Panel G shows gating of the BDCA-3 control sample. Panel H shows gating to include BDCA-3+ cells (MDC2). Because BDCA-3 is also expressed at much lower levels on granulocytes, MDC1 and PDC, only events with a high BDCA-3 fluorescent signal were included in the analysis. DC, dendritic cell; MDC, myeloid DC; PDC, plasmacytoid DC.

were expressed as percentages among total isolated cells (Table 2).

Results from staining for other cell surface markers are also summarized in Table 2.

DC recovery

Recovery of DC was estimated by dividing the number of isolated DC by the number of DC expected to be present in the starting population. The median (range) numbers of BALF cells in the initial samples were 8×10^6 (7×10^5 to 9×10^7). Previous reports suggest that approximately 0.5% of these cells are DC.[12,13] The absolute number of BALF DC expected to be present in the starting population was calculated on the basis of the hypothesis that 0.5% of BALF cells are DC. Following the above method, recovery rates ranged from 0.1 to 4 (median 1.3).

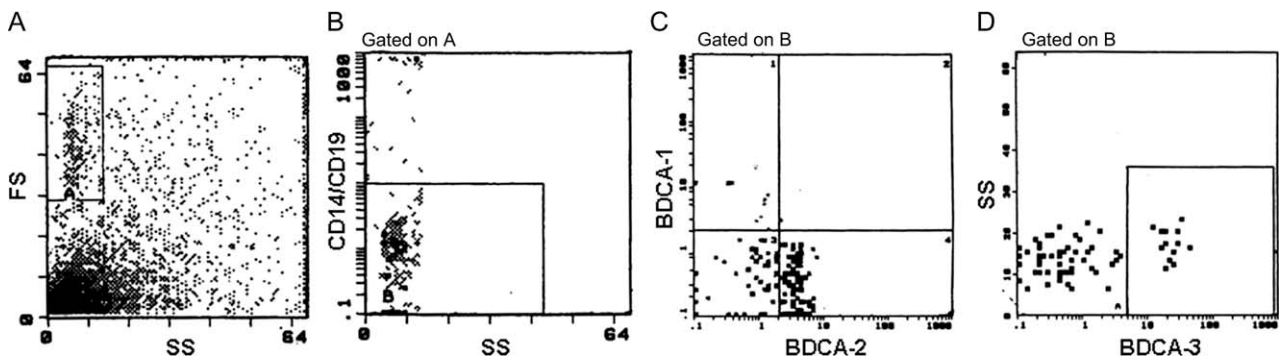

Figure 2 Detection of MDC1, MDC2 and PDC in isolated bronchoalveolar lavage fluid cells after non-DC depletion and BDCA+ cell selection in patient B. Panel A shows gating in forward scatter (FS) and side scatter (SS) modes to exclude dead cells and debris. Panel B shows gating to exclude B cells ($CD19^+$) and monocytes ($CD14^+$). Panel C shows gating to include BDCA-1+ cells (MDC1) and BDCA-2+ cells (PDC). Panel D shows gating to include BDCA-3+ cells (MDC2). DC, dendritic cell; MDC, myeloid DC; PDC, plasmacytoid DC.

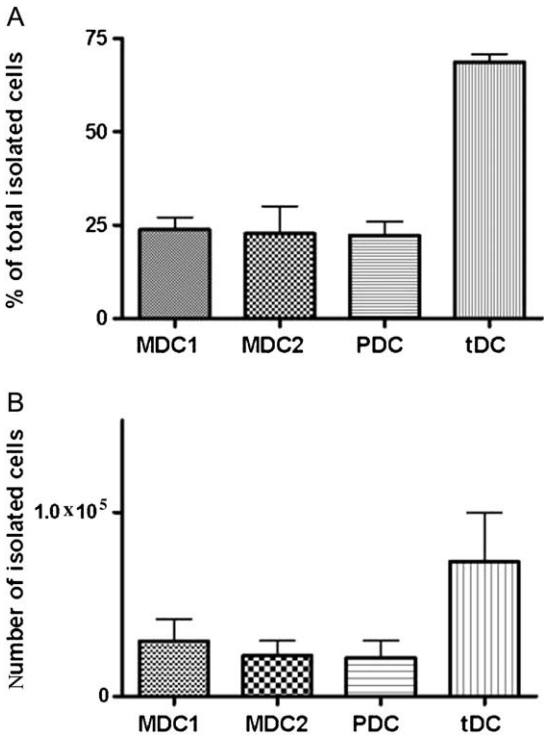

Figure 3 Panel A shows percentages of BDCA-1+ DC (MDC1), BDCA-3+ DC (MDC2), BDCA-2+ DC (PDC) and total DC (MDC1 + MDC2 + PDC = tDC) among total isolated bronchoalveolar lavage fluid cells after non-DC depletion and BDCA+ cell selection. Percentages of tDC represent DC purity of the isolated cell population. Panel B shows the absolute numbers of isolated DC. Results are shown as mean (SEM). DC, dendritic cell; MDC, myeloid DC; PDC, plasmacytoid DC; tDC, total DC.

A more precise estimation of DC recovery would be given by dividing the number of isolated DC by the actual (not the expected) number of DC present in each sample, if this was assessed by flow cytometry. However, BALF DC numbers are low and DC from a sample cannot be enumerated and isolated at the same time.

Capacity of isolated DC to induce naive T-cell responses

The capacity of the isolated BALF DC to initiate T-cell responses was evaluated by coculturing the isolated DC with immunomagnetically sorted CD45RA$^+$ naive T cells. Cultures of naive T cells in the presence of anti-CD3 plus anti-CD28 served as the control. The intracellular cytokine production profile of T cells was evaluated by flow cytometry. Naive T cells cocultured with BALF DC secreted amounts of IFN-γ (median [range], 4.2% [1.7–5.6%]), IL-4 (median [range], 3.6% [0.8–8.5%]) and IL-10 (median [range], 21.3% [9.5, 41%]) similar to those secreted by the anti-CD3 plus anti-CD28 cultures (median [range]: IFN-γ 3.6% [0.4–5%], IL-4 2.3% [0.3–4%] and IL-10 18% [10.8, 47.3%]). Figure 4 shows the results from one of the seven independent experiments carried out.

Table 2 Surface phenotype of cells isolated from bronchoalveolar lavage fluid after non-dendritic cell depletion and BDCA+ cell selection

Surface phenotype	Median (%)	Range (%)
CD14$^-$/CD16$^-$/ILT3$^+$/CD33$^+$	46	26, 64
CD14$^-$/CD16$^-$/ILT3$^+$/CD123$^+$	19	2, 55
HLA-DR	67	59, 74
CD3	0	0, 0
CD14	9	4, 13
CD15	17	11, 25
CD16	19	9, 28
CD19	0	0, 0
CD45	100	99, 100
CD56	1.5	0, 2

Discussion

There is considerable interest in the study of pulmonary DC, as DC have been shown to be the key players in the pathogenesis of common lung disorders, including respiratory infections, lung cancer, asthma and chronic obstructive pulmonary disease (COPD).[4–9] DC can be derived *in vitro* from bone marrow or blood progenitors and matured into various functionally distinct subsets. *In vitro*-derived cells differ phenotypically – and foremost functionally – from pulmonary DC.[10] Techniques for obtaining pulmonary DC from lung surgical resection specimens and BALF have been described in the past. Isolation of BALF DC offers the relative advantage of using a minimally invasive procedure. To date, only BALF CD1a$^+$ DC have been effectively isolated using cell sorting.[13] The technique reported in this study is a new effective, reliable and simple method for isolating three distinct DC subsets from human BALF. Our results suggest that immunomagnetic selection of BDCA positively stained cells can be used to isolate a population of pure and viable DC from human BALF.

This population expressed the DC-specific Ag BDCA-1, BDCA-2 and BDCA-3. One could argue that BDCA-1-positive cells were not DC, because this Ag is also expressed by B cells.[17,22] However, B cells (CD19 positive) were depleted from the initial sample before selecting BDCA-1-positive cells and MDC type 1 were identified as CD19-negative/BDCA-1-positive cells. Accordingly, BDCA-3-positive cells might not be DC, because this Ag is also expressed at much lower levels by monocytes and granulocytes.[17,22] However, granulocytes and monocytes (CD14 positive) were depleted before selecting BDCA-3-positive cells and MDC type 2 were identified as CD14-negative/BDCA-3-positive cells. Moreover, only events with a high BDCA-3 fluorescent signal were included in the analysis.

Another method for the identification of DC is based on the absence of monocyte/macrophage cell markers (CD14/CD16 negative) and the presence of CD85k (ILT3), which is selectively expressed on monocytes, macrophages and DC.[21] Using these phenotypic criteria and CD33 expression to identify MDC or CD123 expression to identify PDC, we confirmed again that our population consisted of DC. To further strengthen the evidence for DC, HLA-DR expression was assessed and we showed that most of the isolated cells were HLA-DR positive. Furthermore, a number of non-DC-lineage markers were used to check for contamination with other cell types (CD3, CD14, CD15, CD16, CD19, CD45, CD56) and results confirmed the presence of only few non-DC types, mainly monocytes, granulocytes and a few NK cells. All these

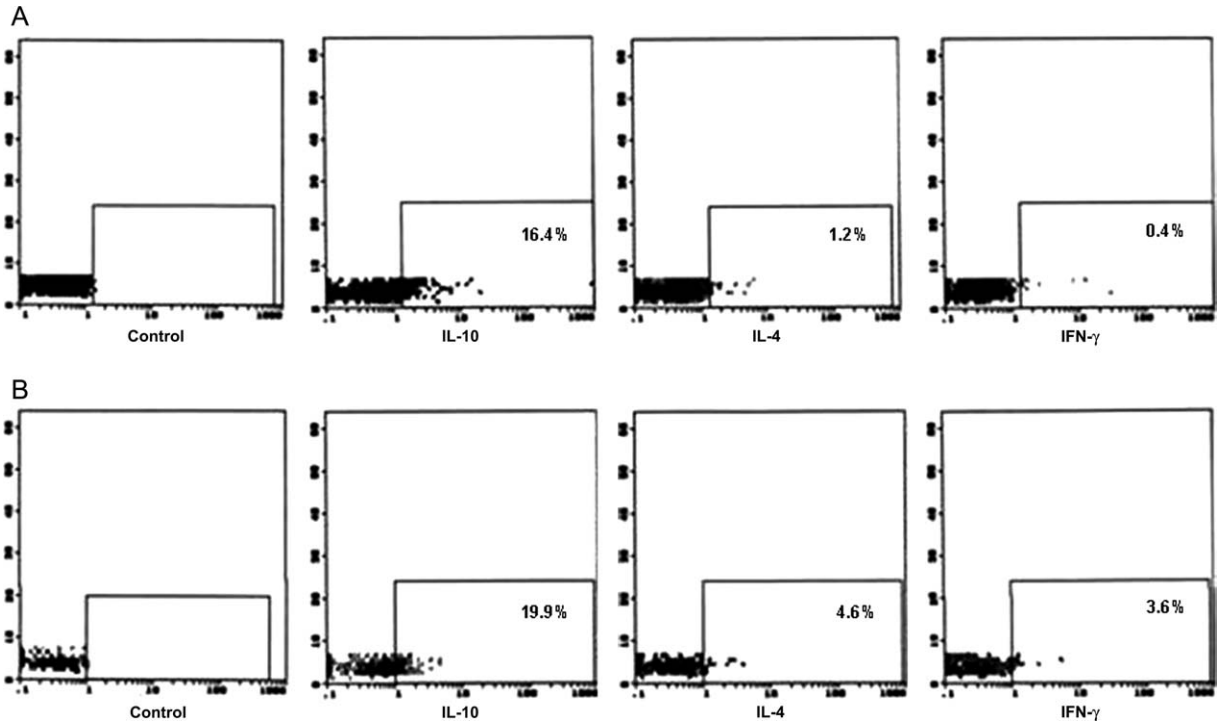

Figure 4 Flow cytometric analysis of intracellular cytokine secretion by naive T cells after culture with anti-CD3 plus anti-CD28 (panel A) or coculture with isolated BALF DC (panel B) for 4 days. BALF DC induced IL-4, IL-10 and IFN production at levels similar to those after anti-CD3 plus anti-CD28 culture. BALF, bronchoalveolar lavage fluid; DC, dendritic cell.

findings suggest that the isolated BALF cell population consisted of true DC.

The functional analysis of the BALF DC obtained by this method compares very favourably with high-purity DC. One of the main characteristics of DC is their striking ability to present Ag to naive T cells and induce T-cell proliferation and differentiation towards type 1 (IFN-γ producing), type 2 (IL-4 producing) or regulatory T cells (IL-10 producing). Critically, we showed the capacity of the isolated cells to induce T-cell responses when compared with naive T cells incubated with anti-CD3 and anti-CD28. This T-cell stimulatory capacity was not a result of the presence of B cells in the isolated cell fraction and could thus only be a result of the presence of DC. Considering that the numbers of cytokine-producing T cells after DC–T-cell coculture were low, this may indicate that BALF DC are not fully mature and would require further activation to induce stronger T-cell responses (Fig. 4).

Previous works by van Haarst *et al.* showed that CD1a-positive DC can be isolated from the low-autofluorescent fraction of BALF cells.[13] However, CD1a-positive cells constitute only part of the total lung DC population. Moreover, some authors call attention to the use of CD1a antibody to purify DC, as it might pre-activate these cells.[23] The technique described in this study offers the advantage of isolation of three distinct DC subsets without using the CD1a antibody. Moreover, the protocol of van Haarst *et al.* requires the use of a FACS, which is not widely available in all clinical research centres.

Demedts *et al.* have only recently introduced the use of BDCA antibodies to label and isolate pulmonary DC from lung resection specimens.[16] According to the protocol followed by these authors, pulmonary mononuclear cells are isolated, T cells ($CD3^+$), monocytes/macrophages ($CD16^+$) and NK cells ($CD11b^+$) are depleted, $CD4^+$ cells are selected (DC express CD4 surface antigen) and pulmonary DC are sorted as low-autofluorescent, CD3/CD19-negative (T and B cells) and BDCA-positive cells. Taken together, results from the study of Demedts *et al.* and ours both indicate that mononuclear cell isolation and lymphocyte and macrophage depletion, followed by selection of BDCA-positive cells, can be commonly applied to isolate pulmonary DC from different types of specimens. In addition, we showed that NK-cell depletion, $CD4^+$-cell selection and cell sorting based on cell autofluorescence could be omitted, at least for isolating BALF DC.

A very interesting new finding is that type 2 MDC (BDCA-3+) are present in human BALF and constitute a significant part of the DC population. A similar finding was reported by Demedts *et al.* in lung resection specimens. Surprisingly, myeloid cells of type 2 are very rare among blood DC (approximately 3% of human blood DC). Although blood MDC type 1, PDC, monocytes and granulocytes also express BDCA-3 at low levels, it is unlikely that other cell types were mistaken as MDC type 2, because only events with a high BDCA-3 signal were included in the analysis and because MDC type 2 were identified as $CD14^-$/BDCA-3+ cells. One possible explanation for the increased numbers of MDC type 2 is that many specimens were taken from patients with lung cancer, which might have caused accumulation of these cells in the lung.

MDC type 2 is a novel subset of MDC expressing high levels of the BDCA-3 Ag. With respect to phenotype, morphology, endocytic capacity and maturation requirements, this DC subpopulation is quite similar to MDC type 1.[17] However, there are some striking differences, such as a lack of Fc receptor

expression.[17] The lack of Fc receptor indicates that MDC type 2, unlike type 1, do not have the capacity of Ig-mediated Ag uptake. It is possible that recruitment of MDC type 2 in the lung might be related to a reduced capacity to recognize pathogens or cancer cells. This is an intriguing hypothesis that might be worthy of further investigation.

There are certain limitations to our study. First, due to the scarcity of DC in human BALF, a large volume of BALF is required to isolate a sufficient number of DC (at least 25 mL). This makes our technique unsuitable for paediatric subjects. Second, recovery rates were only roughly estimated on the basis of previous studies on BALF, which showed that approximately 0.5% of BALF cells are DC.[12,13] However, there are subjects with higher or lower percentages of BALF DC. This might explain why recovery rates were variable and in some cases higher than 1. Third, the procedure is time-consuming and expensive. Fourth, one could argue that our technique fails to isolate DC expressing CD1a but not BDCA-1 (CD1c). This might be true for blood DC, where $CD1c^+$ cells do not express CD1a, but in the human lung most $CD1c^+$ cells co-express CD1a.[16,17] Finally, further functional experiments to ascertain the identity of DC, such as showing IFN-α production by PDC after CpG stimulation, were not carried out and should be considered in future studies.

In summary, the results of this study showed that immunomagnetic cell separation can be a reliable and simple method to isolate functionally intact BALF DC that show a strong capacity to induce immune responses. The technique that we propose enabled for the first time the isolation of MDC type 2 and PDC from human BALF. Surprisingly, we showed that the rare subpopulation of MDC type 2 can be found to be exceedingly increased in the lungs. Considering that isolation of BALF DC provides a convenient and efficient way to study the properties and functional capacities of pulmonary DC, this method is expected to help elucidate the important role of DC in various pulmonary disorders. Moreover, it would be reasonable to assume that the viable and functional BALF DC population obtained by our protocol may be suitable for the development of immunotherapeutic vaccines.

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