

Identification and Characterization of Human Pulmonary Dendritic Cells

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Dendritic cells (DC) are specialized antigen-presenting cells, linking innate and adaptive immune responses, and thus play an important role in immunologically mediated diseases, including pulmonary diseases such as asthma and respiratory viral infections. Although much is known about the characteristics of lung DC in animal models, very few data concerning human lung DC are available. The goal of our study was to identify and characterize dendritic cells in human lung by preparing single-cell suspensions from surgical resection specimens and subsequent labeling with the recently developed blood dendritic cell antigen (BDCA) markers. A straightforward isolation procedure was developed to avoid phenotypical and functional changes induced by extensive purification methods. In this way, human lung DC were directly identified without the need for an additional adherence step for further purification. For the first time, we demonstrate the presence of three previously unidentified DC subsets in human lung digests: myeloid DC type 1 (BDCA1+/HLA-DR+), myeloid DC type 2 (BDCA3+/HLA-DR+), and plasmacytoid DC (BDCA2+/CD123+). The presence of CD1a+ DC in the human lung was confirmed. The identification and characterization of different human pulmonary DC subtypes is of great importance for the future development of DC-based immunotherapies.

Keywords: dendritic cells; human lung; BDCA

Dendritic cells (DC) are antigen-presenting cells (APC) that play a crucial role in the initiation and modulation of an appropriate response of our immune system to danger signals (bacteria, viruses, etc.) by linking innate to adaptive immune responses (1). They have unique functional and morphologic properties: compared with other APC such as macrophages and B cells, they are much stronger T cell stimulators and can be morphologically distinguished by their typical cytoplasmatic extensions ("dendrites"). DC are recruited from the blood circulation to peripheral organs, where they continuously sample their environment for foreign substances. They are able to take up and process antigens and migrate to secondary lymphoid organs where they present the processed antigen to T cells and initiate an adaptive immune response (2). It is clear that disturbances of this tightly regulated mechanism can lead to an inappropriate reaction of the immune system and can cause different pathologic conditions. The role of DC in immunologically mediated diseases (AIDS, allergy, cancer, etc.) has been extensively studied, which has not only provided new insights into the pathogenesis of those diseases, but also paved the way for new therapeutic strategies (3).

In mice, pulmonary DC have been shown to be the key cells in the pathogenesis of asthma (4), and there is circumstantial

evidence from animal models that pulmonary DC might play a role in the development of chronic obstructive pulmonary disease (COPD) in smokers (5). Moreover, DC mediate protection against pulmonary tuberculosis infection in mice (6, 7) and elevated numbers of DC have been detected in lung cancer tissue in humans (8). Furthermore, the number of lung DC is increased in sarcoidosis (9) and diffuse panbronchiolitis (10). These data clearly demonstrate that pulmonary DC are at least involved in the pathogenesis of a whole spectrum of highly prevalent respiratory diseases and that for some of those (such as asthma), DC have been proven to be essential in the development of the disease (3).

These findings push the need for a thorough investigation into the human pulmonary DC. Although the characteristics of lung DC have been extensively studied in animal models, only limited data are available concerning the human pulmonary DC. In the past, a few groups studied the presence of APC in the human lung (11–13), but their studies were hampered by the lack of DC-specific markers or by rather extensive isolation and purification methods (e.g., overnight incubation for enrichment of transiently adherent mononuclear cells). These extensive purification procedures could induce phenotypical and functional changes in DC and thus might yield important differences between the isolated pulmonary DC and the DC as it is actually present in the human lung. To avoid such artificial alterations in DC phenotype and function, we developed a new protocol for the identification of human lung DC in surgical resection specimens in which overnight incubation as an enrichment step could be omitted. Subsequently, we used recently developed markers for human blood DC (14) to determine the presence of DC in human lung. Three new pulmonary DC subpopulations could be identified, while the presence of a fourth subtype (CD1a+ epithelial DC) was confirmed.

MATERIALS AND METHODS

Reagents and Media

Ficoll-Paque was obtained from Amersham Pharmacia (Uppsala, Sweden). Tissue culture medium (TCM) was prepared using RPMI-1640 supplemented with 5% FCS, penicillin/streptomycin, L-glutamine, and 2-mercaptoethanol (all from GibcoBRL, Carlsbad, CA). For mixed leucocyte reactions, TCM supplemented with 10% human AB serum instead of FCS was used. Digestion medium consisted of TCM supplemented with 1 mg/ml collagenase type 2 (Worthington Biochemical Corp., Lakewood, NJ) and 0.02 mg/ml DNase I (grade II from bovine pancreas; Boehringer Mannheim, Brussels, Belgium). "FACS-EDTA" buffer contained PBS w/o Ca²⁺ or Mg²⁺, 0.1% azide, 1% bovine serum albumine (BSA; Sigma, St. Louis, MO) and 5 mM EDTA.

Preparation of Single-Cell Suspensions from Lung

Lung tissue was obtained from patients who underwent lobectomy or pneumectomy for various reasons (mostly lung cancer). Written informed consent was obtained from all subjects according to protocols approved by the Medical Ethical Committee of the Ghent University Hospital. Tissue distant from the primary pathologic lung tissue was collected by a pathologist. Resection specimens were rinsed with TCM to remove residual blood. If possible, the pulmonary circulation was flushed with TCM to minimize contamination with blood. Lung tissue

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was minced using scissors and incubated in digestion medium in a humidified incubator at 37°C and 5% CO₂. After 30 min incubation, samples were resuspended, fresh digestion medium was added, and incubation continued for another 15 min. Subsequently, after centrifugation, samples were resuspended in Ca²⁺ and Mg²⁺-free PBS containing 10 mM EDTA for 5 min at room temperature on a shaker. Next, enzyme-digested fragments were passed through a 40-μm cell strainer (Becton Dickinson Labware, Bedford, MA) and subsequently separated on a Ficoll density gradient to obtain pulmonary mononuclear cells (PMC). Finally, samples were subjected to RBC lysis, washed in FACS-EDTA, and kept on ice until immunofluorescent labeling.

Labeling of Single-Cell Suspensions for Flow Cytometry

Single-cell suspensions were pre-incubated with human IgG to reduce nonspecific binding. Monoclonal antibodies that were used included: FITC-conjugated anti-lineage (lin) markers cocktail, anti-CD3 (clone UCHT1), -CD14 (M5E2), -CD19 (HIB19); phycoerythrin (PE)-conjugated anti-CD1a (HI149), -CD14 (M5E2), -CD16 (3G8), -CD54 (HA58), -CD123 (9F5), CD11c (B-ly6), -CD40 (5C3), -CD80 (L307.4), -CD86 (FUN-1), -HLA-DR (G46-6), -CD83 (HB15e), -CD207 (Langerin, clone DCGM4), and PE-conjugated isotype controls mouse IgG1, IgG2a, and IgG2b; and allophycocyanin (APC)-conjugated anti-CD1c (BDCA1, clone AD5-8E7), -BDCA2 (AC144), and -BDCA3 (AD5-14H12), and APC-conjugated isotype controls mouse IgG1 and IgG2a. All monoclonal antibodies were obtained from BD-Pharmingen (Erembodegem, Belgium) except PE-conjugated anti-CD207 (Immunotech, Marseille, France) and APC-conjugated anti-BDCA1 (Blood Dendritic Cell Antigen 1), -BDCA2, -BDCA3, and -BDCA4 (all from Miltenyi Biotec, Bergisch Gladbach, Germany).

Cell Analysis and Sorting

Flow cytometric data acquisition was performed on a FACS Vantage SE equipped with 488 nm and 633 nm lasers and running CELLquest 3.3 software (Becton Dickinson, San Diego, CA). FlowJo software (www.treestar.com/flowjo) was used for data analysis on PowerMac G3 and G4 workstations (Apple Computer Inc., Cupertino, CA). For detection of DC, different negative gate settings were tested to exclude cell types other than DC from analysis. Autofluorescence (detected in the FL1 channel) was used to exclude macrophages, while in some experiments, additional markers were added in the FL1 channel to exclude lin⁺ (CD3, CD19, CD20, CD14, CD16, CD56) cells or the combination of CD3⁺ and CD19⁺ cells. Different strategies to identify DC were evaluated and are described extensively below.

For sorting experiments, cells were sorted according to autofluorescence properties—high autofluorescent (HAF) cells versus low autofluorescent (LAF) cells—or additional markers were used to eliminate T and B cells from the LAF fraction, creating a population of LAF, CD3[−], CD19[−] cells on the one hand and a mixed population of HAF, CD3⁺ and CD19⁺ cells on the other hand. Cyto-centrifuge preparations were prepared from freshly sorted LAF and HAF cells and stained with May-Grunwald-Giemsa reagent for morphologic analysis.

Isolation of Peripheral Blood T Cells

T cells were isolated from human peripheral blood mononuclear cells (obtained after Ficoll density gradient on blood obtained from healthy donors) by magnetic depletion of non-T cells using a commercially available Pan T Cell Isolation kit (Miltenyi Biotec).

Isolation of BDCA⁺ Lung Cells

BDCA⁺ cells were purified from PMC using a commercially available isolation kit (Miltenyi Biotec). Briefly, PMC were first depleted of T cells, monocytes/macrophages, and natural killer (NK) cells using anti-CD3, -CD11b, and -CD16 beads. Next, this depleted population was incubated with anti-CD4 beads and CD4⁺ cells were retained. Because all BDCA⁺ cells express CD4, this method allows a pre-enrichment of BDCA⁺ cells and avoids contamination with lymphocytes, monocytes/macrophages, and NK cells. After this pre-enrichment procedure, cells were labeled and sorted using the following criteria: LAF, CD3[−]CD19[−], and BDCA⁺. In our hands, this resulted in a BDCA⁺ lung cell population of ± 90% purity (cells positive for BDCA1, BDCA2, or BDCA3).

Mixed Leukocyte Reaction

Stimulator cells were LAF and HAF cells sorted as described above. In a separate experiment, T cell stimulatory capacity of LAF/CD3[−]/CD19[−] cells was compared with the group of HAF, CD3⁺, and CD19⁺ cells. Purified allogeneic T cells were cocultured in round-bottom 96-well plates (in duplicate or triplicate) with a serial dilution of stimulator cells for 5–6 d at 37°C. Cells were pulsed with ³H-thymidine for 16 h before being harvested. Cell proliferation was assessed on an automated liquid scintillation counter (Microbeta, Turku, Finland).

Immunohistochemistry

To evaluate the presence and distribution of CD1a⁺ and Langerin⁺ cells in human lung, a series of acetone fixed cryosections was stained using an automated staining procedure (Ventana iView Detection system; Ventana Medical Systems, Tucson, AZ): at first, sections were incubated with the primary antibody for 1 h, followed by biotinylated goat anti-mouse IgG. Finally, slides were incubated with streptavidin horseradish peroxidase and colored with diaminobenzidine. Primary antibodies used were mouse anti-human CD1a (DakoCytomation, Heverlee, Belgium) and mouse anti-human Langerin (Immunotech).

RESULTS

Identification of Different DC Subpopulations in Human Lung

A major finding in this study is the observation of three previously unidentified DC subsets in human lung digests: CD1c (= BDCA1)⁺/HLA-DR⁺, BDCA2⁺/CD123⁺, and BDCA3⁺/HLA-DR⁺ lung DC. Lung DC were defined using different exclusion criteria: in a first series of experiments, HAF cells were excluded from analysis, based on previous studies on human bronchoalveolar lavage (BAL) (15) and lung digests (16) that identified HAF cells as being almost exclusively macrophages. These findings were confirmed in our analyses: freshly isolated pulmonary mononuclear cells were sorted on the base of autofluorescence and a morphologic comparison between HAF and LAF cells was made. HAF cells (Figure 1A) appeared to

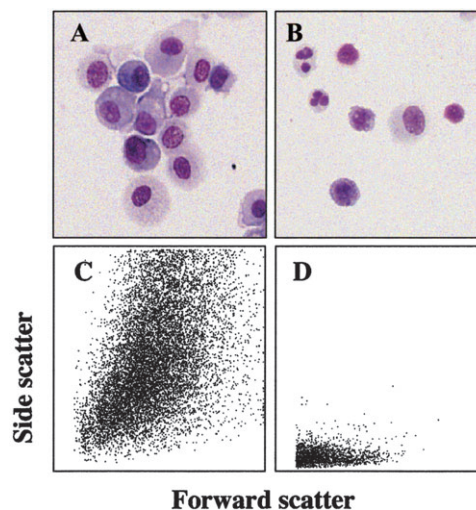


Figure 1. Morphologic comparison between pulmonary mononuclear cells freshly sorted according to autofluorescence properties. Photomicrographs of cyto-centrifuge preparations stained with May-Grunwald-Giemsa reagent. High autofluorescent (HAF) cells (A) display a typical macrophage-like morphology, whereas low autofluorescent (LAF) cells (B) have a heterogeneous morphology. Macrophages (C) have a higher side scatter (reflecting higher granularity and complexity) as well as a higher forward scatter (reflecting cell size) compared with dendritic cells (DCs) (D).

be large rounded cells with oval or round nuclei and vacuolar cytoplasm (a typical morphology of macrophages), whereas LAF cells were smaller and displayed a heterogeneous morphology (Figure 1B).

These findings justify the first gating strategy for the detection of DC in human lung digests, where HAF cells were excluded from analysis and DC were identified in the LAF fraction by using the following staining combinations: CD1c (BDCA1)+/HLA-DR+, BDCA2+/CD123+, and BDCA3+/HLA-DR+. The blood dendritic cell antigen (BDCA) markers are recently developed markers specific for DC in human blood (14), where they discriminate between three different blood DC subsets: myeloid DC type 1 (MDC1, which are BDCA1+), myeloid DC type 2 (MDC2, which are BDCA3+), and plasmacytoid DC (PDC, which are BDCA2+/CD123+). However, the presence of BDCA+ cells in human lung digests has never been studied before, and although earlier studies on human lung DC were, as mentioned before, hampered by the lack of specific markers, this problem is now mainly overcome by the availability of these new BDCA antibodies. Using these different staining combinations, all three different DC subsets could consistently be identified in human lung digests (Figure 2A), representing a small but significant fraction of total PMC.

Differences in Negative Gate Settings

By using HAF as an exclusion criterium, DC can be distinguished from macrophages. However, one could argue that BDCA markers might be expressed in the lung on cell types other than DC, and that low autofluorescent BDCA+ cells are falsely consid-

ered to be DCs. This is especially the case for myeloid DC (MDC1 and MDC2). Indeed, BDCA1 is also expressed on a minor subset of human B lymphocytes, whereas BDCA3 is expressed at low level on CD14+ monocytes in peripheral blood. BDCA2, on the other hand, is strictly expressed on human plasmacytoid DC and co-expression of BDCA2 and interleukin-3 receptor (CD123) is very straightforward for the identification of PDC.

To avoid the possibility of contamination of the BDCA+ cells with non-DC cell types, a second gating strategy was developed where an additional negative gate was defined and all lin+ cells (CD3, CD14, CD16, CD19, CD20 or CD56+ cells) were excluded from analysis for human lung DC, in addition to the exclusion of macrophages. By using this approach, the previously identified lung DC subpopulations could still be detected without any doubt on possible contamination with other cell types (Figure 2B). Using this gating strategy, respiratory DC are defined as low autofluorescent, lin- and BDCA+. On the one hand, this gating strategy is very strict and by using surface markers for the exclusion for T- and B-lymphocytes, monocytes, granulocytes, and NK-cells, one can be sure that the lin-BDCA+ cells are indeed pure dendritic cells. On the other hand, this strategy might be all too vigorous and by eliminating all lin+ cells, a number of DC is probably excluded from analysis. Indeed, it has been shown that some BDCA1+ cells in blood are weakly positive for CD14 and that there might be some CD56 expression on a small subset of BDCA1+ as well as on BDCA3+ blood DC's. Additionally, it has been demonstrated previously that CD14 and CD16 are expressed on DC precursors in blood and are downregulated when those precursors develop to fully mature DC (17, 18). So by excluding CD14+ and CD16+ cells from analysis, one could underestimate the total DC population present in human lung, because a number of pulmonary DC (most probably myeloid DC at an immature or precursor stage) might still have some CD14 or CD16 expression.

Taking these elements in consideration, a third gating strategy was evaluated, in which high autofluorescent cells (macrophages), CD3+ (T-lymphocytes) and CD19+ cells (B-lymphocytes) were excluded from analysis and BDCA expression was evaluated in the LAF/CD3-/CD19- fraction of pulmonary mononuclear cells (Figure 2C). This is a less stringent approach than eliminating all lin+ cells, but still adds some value to the first gating strategy in which only macrophages are omitted. By using this strategy, one looks for BDCA expression on a mixed population of monocytes and DC and cells that express BDCA are considered to be DC. By doing so, some monocytes might be falsely classified as DC. This is not the case for PDC and MDC1 (neither BDCA1 nor BDCA2 are expressed on monocytes), but might be a problem for the identification of MDC2 since BDCA3 is expressed at low levels on monocytes. However, by using high HLA-DR expression as an additional criterium, one can conclude that low autofluorescent, CD3-/CD19-, BDCA1+/HLA-DR+, and BDCA3+/HLA-DR+ cells are true myeloid dendritic cells, whereas plasmacytoid DC are defined as LAF, CD3-/CD19- and double positive for BDCA2 and CD123. We propose this method as the best way to identify DC in human lung, and by using this strategy the presence of three different DC subtypes in human lung could be demonstrated for the first time: MDC1, MDC2, and PDC. These different subsets could consistently be identified in all resection specimens at low numbers: mDC1 1.18% of lung PMC (± 0.19 SEM), mDC2 1.91% (± 0.27 SEM), and pDC 0.57% (± 0.1 SEM).

Scatter Profile of Human Lung DC and Macrophages

As described above, microscopic examination shows that HAF cells are quite large cells compared with the LAF cells (Figure 1).

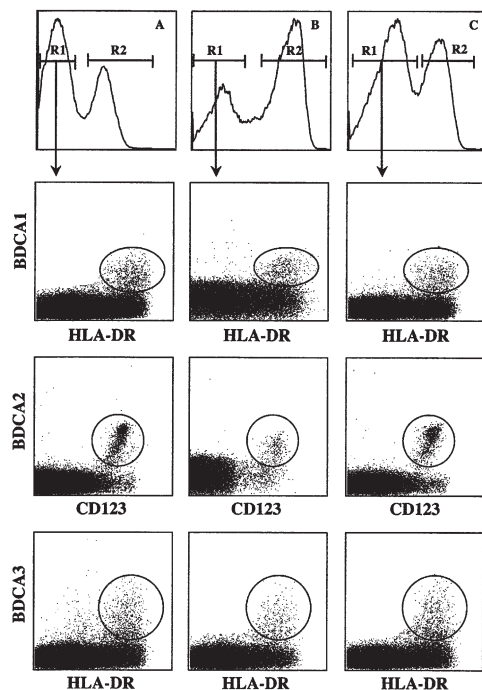


Figure 2. Identification of DC in human lung digests using different gating strategies. At first (A), DC were defined in the LAF population (R1) as BDCA1+/HLA-DR+ (MDC1), BDCA2+/CD123+ (PDC), or BDCA3+/HLA-DR+ (MDC2), whereas HAF cells (R2) were excluded for analysis. A second gating strategy (B) excluded all lin+ cells (R2), and DC were identified in the LAF, lin- fraction (R1). In a final approach (C), HAF, CD3+, or CD19+ cells (R2) were omitted, and BDCA-expression was evaluated in the LAF, CD3-, CD19- fraction (R1).

This is reflected in a high forward scatter (which is a measure for cell size) when analyzed by flowcytometry (Figure 1C). The different respiratory DC subtypes all have a much lower forward scatter (Figure 1D), which could be already be expected from the microscopic analysis of LAF cells, that appeared to be predominantly small cells. Side scatter profiles (a measure for cell granularity and complexity) of respiratory DC are also much lower than that of macrophages. This demonstrates that, in addition to the amount of autofluorescence, one can use the scatter profile to differentiate between macrophages and DC. This is not needed when using DC-specific markers (such as the BDCA markers), but can be of use when surface markers are used that are commonly expressed on macrophages and DC, such as HLA-DR and CD11c. This staining combination is often used to identify DCs in blood, but cannot discriminate between lung DC and macrophages because the latter also express both of these markers.

Functional Properties

One of the main characteristics of DC is their striking ability to stimulate the proliferation of T cells, and in a much stronger way than other APC such as B cells and macrophages. This typical functional property of DC was evaluated by mixed leukocyte reaction to confirm that the applied gating strategy is indeed appropriate to identify DC in human lung. At first, the T cell stimulatory capacity of freshly sorted LAF cells was compared with that of HAF cells, which consisted predominantly of macrophages, as described earlier. The LAF cells appeared to have much stronger T cell stimulatory capacity than HAF cells (Figure 3A), a finding consistent with previous studies performed by other groups (16). However, one might still argue that the T cell stimulation in the LAF fraction is due to the presence of B lymphocytes. To check for this possibility, CD3-CD19-LAF cells were incubated with allogeneic T cells and the extent of T cell stimulation was compared with that of the combined group of HAF, CD3+, and CD19+ cells. On the one hand, this approach allows to exclude T cell stimulation by B lymphocytes in the LAF group, while on the other hand it offers the ability to check the validity of the proposed method for the identification of respiratory DC: to justify the applied gating strategy, it should be demonstrated that the T cell stimulatory capacity of the LAF, CD3-CD19- cells (the fraction wherein BDCA+ cells are defined as DC) is stronger than that of their counterparts (the combined group of macrophages, T, and B-lymphocytes) that are excluded for analysis on BDCA expression. The functional comparison of both fractions (Figure 3B) clearly shows the stronger T cell stimulatory capacity of LAF, CD3-CD19- cells, which can only be due to the presence of DC in this fraction.

A definite proof of the presence of DC in human lung would be to purify BDCA+ cells from human lung and to investigate their functional properties. If the BDCA+ cells are true DCs, they should be able to stimulate T cell proliferation vigorously. To evaluate this, BDCA+ cells were purified from human lung using a commercially available isolation kit (as described above) and their capacity to stimulate T cell proliferation was compared with that of macrophages. The results of this approach demonstrate the strong T cell stimulatory capacity of BDCA+ lung cells and confirm that these cells are indeed true dendritic cells (Figure 3C). Conclusively, we first demonstrated the T cell stimulatory capacity of LAF lung cells; then described that this was not merely due to the presence of B cells and finally showed the strong T cell stimulatory capacity of BDCA+ lung cells, which are thus beyond any doubt respiratory dendritic cells. It would be very interesting to compare the functional properties of the different DC subpopulations in human lung in future

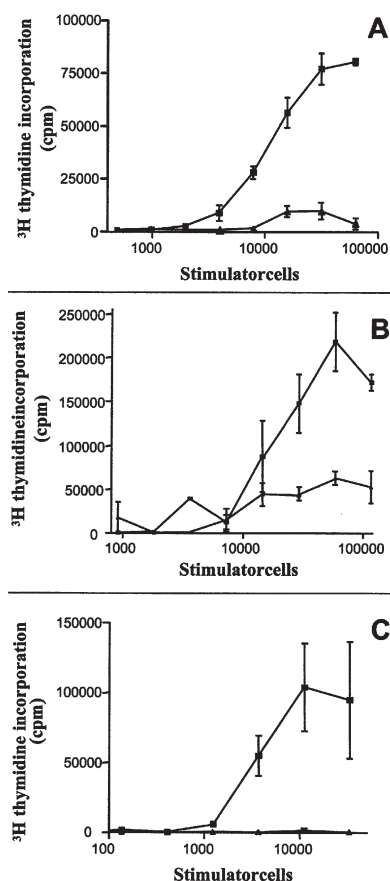


Figure 3. Comparison of functional properties of different pulmonary mononuclear cell subpopulations. At first (A), freshly sorted LAF and HAF cells were incubated with allogeneic T cells in a primary allogeneic mixed leukocyte reaction and T cell proliferation was measured through the incorporation of ^3H thymidine. LAF cells (squares) clearly demonstrate a much higher T cell stimulatory capacity compared with HAF cells (triangles). Next (B), freshly sorted LAF, CD3-CD19- cells (squares) were compared with a mixed population of HAF, CD3+, and CD19+ cells (triangles), where the former appeared to be the strongest inducers of T cell proliferation. Finally (C), purified BDCA+ lung cells (squares) were shown to have a very strong capacity to stimulate T cell proliferation, especially when compared with macrophages (triangles).

experiments to further unravel their different roles in pulmonary immune responses.

Phenotypic Characterization of Different Respiratory DC Subsets

The identification of three previously unknown pulmonary DC subsets in lung digests provokes many questions and much speculation on their possible divergent roles in physiologic and pathologic conditions in human lung, and on how these different subsets might influence the course of common respiratory diseases. In a first attempt to explore the differences between these DC subsets, phenotypical characteristics of lung DCs were evaluated. This might not only provide a clue to distinctive functional properties, but is also of great importance in view of the possible use of lung DC as therapeutic targets in the future.

In a first series of experiments, the expression of some markers present on DC precursors in blood was evaluated on pulmonary DC (Figure 4). This might add some information on the developmental origin of the respiratory DC subsets, and allows a comparison between the different respiratory DC subtypes and their counterparts in peripheral blood. The strategy to evaluate the expression of these markers was as follows: non-DC were gated out using HAF, CD3+, or CD19+ as an exclusion criterion and subsequently, all cells positive for a DC-specific marker (BDCA1, BDCA2, BDCA3) were analyzed for the expression of a marker of interest and compared with an isotype staining control for this marker. There was a very clear difference in the expression of CD11c, CD14, and CD16 between the different subgroups of respiratory DC. All BDCA1+ DC (MDC1) express CD11c at high levels, whereas BDCA2+ DC (PDC) do not express CD11c. The majority of BDCA3+ DCs (MDC2)

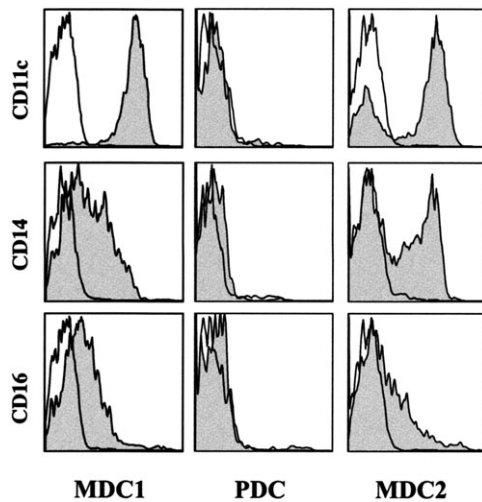


Figure 4. Expression of lineage markers on different lung DC subsets. Expression of these surface markers was evaluated on LAF, CD3–CD19– cells that were positive for BDCA1 (MDC1), BDCA2 (PDC), or BDCA3 (MDC2). Gray histograms indicate specific marker; white histograms indicate isotype control staining.

strongly express CD11c, but there is a small population that is CD11c–. These findings are roughly similar to the CD11c expression of blood DC, where MDC1 are CD11c^{high}, MDC2 are CD11c^{dim}, and PDC do not express CD11c. However, as mentioned above, a small population of BDCA3+ DCs (MDC2) in lung was CD11c–. A possible explanation for this finding is the fact that BDCA3 is also expressed at low level on PDC (A. Dzionek, personal communication), which might thus represent the BDCA3+CD11c– population.

As for the expression of CD14, the expression pattern is different on all three DC subsets. BDCA1+ DC have a heterogeneous expression of CD14, with some cells expressing very few or no CD14 at all, and other BDCA1+ DC expressing CD14 at low level, while lung PDC have no CD14 expression. For the BDCA3+ lung DC, there seem to be two clearly distinct subgroups regarding to CD14 expression. One subgroup has no CD14 expression at all, whereas the other subgroup does express CD14 at significant levels.

Regarding CD16 (FcγRIII), there was no expression on lung PDC, but a heterogeneous expression of CD16 on MDC1 as well as on MDC2 was observed.

In addition to these lineage markers, expression of maturation markers CD40, CD80, CD86, intercellular adhesion molecule 1 (ICAM-1, identical to CD54), and HLA-DR was investigated and compared between pulmonary MDC1, MDC2, and PDC (Figure 5). There is a striking difference in maturation status between PDC and MDC. Lung PDC have almost no expression of maturation markers, whereas MDC (MDC1 as well as MDC2) seem to be more mature because they express CD40, CD80, CD86, CD54, and HLA-DR at higher levels than PDC. It has to be mentioned that HLA-DR expression on pDC, although at lower levels than on mDC, is rather high when compared with the expression of other maturation markers on pDC. This finding however was seen in all samples tested, with a mean MFI ratio of 47 (± 11 SEM) for intensity of HLA-DR expression on pDC when compared with isotype staining control.

Confirmation of the Presence of CD1a+ DC in Human Lung

There is some controversy on the presence of CD1a+ DC in human lung: some authors (12) did not find any CD1a+ cells

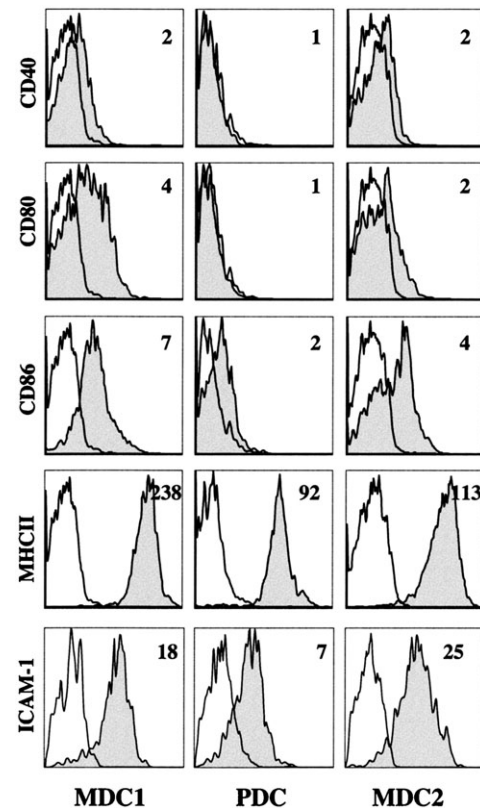


Figure 5. Expression of markers involved in antigen presentation and T cell costimulation on different lung DC subsets. Expression of these surface markers was evaluated on LAF, CD3–CD19– cells that were positive for BDCA1 (MDC1), BDCA2 (PDC), or BDCA3 (MDC2). Gray histograms indicate specific marker; white histograms indicate isotype control staining. The numbers indicate mean fluorescence intensity relative to isotype control.

in human lung, some demonstrated that the presence of CD1a+ DCs was restricted to bronchial (sub)epithelium (11), and others described the presence of CD1a+ DCs not only in epithelium but also in lung parenchyma (19). To evaluate the presence of CD1a+ DCs in human lung, the same method as for the BDCA+ DCs was applied. CD1a+ DC could consistently be identified in human lung digests as LAF, CD3–CD19–, CD1a+HLA-DR+ cells. On a few occasions, expression of Langerin (CD207) on CD1a+ DCs was investigated and, consistent with previous findings on CD1a+ DC in skin and lung (20), CD1a+ DCs in human lung also appeared to express Langerin. To evaluate the distribution of CD1a+ DC in human lung, immunohistochemical analysis of surgical resection specimens was performed. CD1a+ DC were found mainly in the epithelia of large (cartilaginous) as well as of smaller (noncartilaginous) airways, whereas no CD1a+ cells could be demonstrated in lung parenchyma (Figure 6A). This distribution pattern was the same as for Langerin+ cells, which could also be demonstrated in the respiratory epithelium of the conducting airways, but not in lung parenchyma (Figure 6B).

Coexpression of CD1a and CD1c on Lung DC

Although the presence of BDCA1+/HLA-DR+ cells in human lung digests has not been described before, there are some interesting data regarding this cell population obtained from immunohistochemical studies on human lung tissue. BDCA1 is identical to CD1c and the presence of CD1c+ cells in human lung has

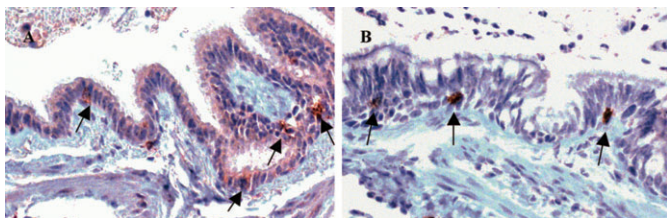


Figure 6. Immunohistochemical analysis for CD1a (A) and Langerin (B) expression in sections of human lung tissue. CD1a+ cells can be found in the epithelium of conducting airways (A), but not in lung parenchyma (data not shown). The same applies for Langerin+ cells (B), whose presence is also limited to the airway epithelium.

been described by a few groups (10, 19, 21). CD1c+ DC appear to be closely related to CD1a+ DC: they are both present in bronchial epithelium as well as in the submucosa, although it seems that the majority of CD1a+ DC is located in the epithelium, whereas CD1c+ DC are more abundant in the submucosa. By using FACS analysis, we evaluated the co-expression of CD1a and CD1c (BDCA1) on lung DC to investigate the possibility that this is actually one and the same population. We found that the latter is not the case, and that while the majority of CD1c+ DC also express CD1a, separate small populations of CD1c+ CD1a- and CD1c-CD1a+ DC could consistently be identified (Figure 7). There is no expression of CD1a on mDC2 and pDC in human lung (Figure 7).

DISCUSSION

Although the characteristics and functional properties of lung DC are extensively studied in animal models, only very few data concerning their human counterparts are available. This is mainly due to the poor availability of tissue (especially when compared with animal models) and to the very labor-intensive and time-consuming experimental setup, whereas an additional problem has been the lack of specific markers for DC in the past. However, the presence of accessory cells has been described in the LAF of mononuclear cells derived from human lung digests (13, 16) as well as in the LAF population of cells obtained by BAL in human subjects (15). These findings are crucial for the further study of DC in human lung. Still, a few considerations have to be taken into account when interpreting these data. First, these studies demonstrate beyond any doubt the presence of potent accessory cells in human lung, but a detailed characterization of this population has been hampered by the lack of DC-specific markers. In addition, it is very important to remember the extensive isolation procedure used in these studies: using an overnight culture step to isolate the transiently adherent

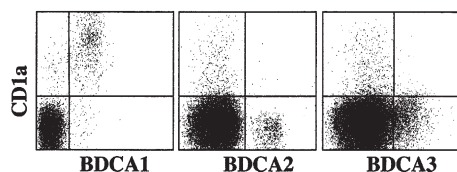


Figure 7. Expression of CD1a on different lung DC subsets. Dot plots shown are gated on LAF, CD3-CD19- cells. The majority of mDC1 expresses CD1a. However, BDCA1+CD1a- and BDCA1-CD1a+ could consistently be identified at low numbers. pDC and mDC2 do not express CD1a.

mononuclear cells (this is a way of enriching a cell population for DCs) might induce phenotypical and functional changes in these cells. DC are very sensitive to such *in vitro* manipulations, so one always has to keep in mind that the characteristics of the isolated DC do not necessarily reflect the actual state of the DC *in situ*. Finally, analysis of DC in BAL, although of extreme importance, is limited to a selected compartment of the human lung (airway lumen and alveolar spaces).

To avoid most of these problems, we developed a protocol in which the isolation procedure could be limited to the lung digest and a density gradient procedure to obtain pulmonary mononuclear cells, while an additional overnight culture step could be omitted. By using recently developed DC specific surface markers, we managed to identify three different respiratory DC subsets in lung digests: MDC1 (BDCA1+/HLA-DR+), MDC2 (BDCA3+/HLA-DR+), and pDC (BDCA2+/CD123+). These DC subtypes can consistently be found at low numbers in human lung digests. Several findings point out that these cells are indeed true DC. At first, to phenotypically define a dendritic cell, the combination of at least two surface markers was used, and one of those (the BDCA marker) was specific for DC. Using this double positivity strengthens the evidence for the presence of DC in human lung. However, the BDCA markers are developed for the identification of DC in human blood, and one could still argue that BDCA+ lung cells are actually macrophages (which are not present in the blood) or other leukocytes that acquired BDCA expression during their migration from the circulation to the lung. To check for this possibility, we excluded HAF cells (macrophages) and all cells that were positive for a non-DC lineage marker (CD3, CD14, CD16, CD19, CD20, CD56), and still BDCA1/HLA-DR+, BDCA2+/CD123+, and BDCA3+/HLA-DR+ cells could consistently be found in this LAF, lin- population, clearly showing that these are indeed DCs.

In addition to this very tight phenotypical characterization, functional criteria have to be fulfilled to identify dendritic cells. Indeed, one of the main characteristics of DC is their striking ability to induce T cell proliferation, probably the most important criterion to define a DC. First, we demonstrated the strong capacity of LAF lung cells to induce T cell proliferation when compared with HAF cells (macrophages). This confirms the earlier findings by other groups and justifies the search for DC in this LAF population. Next, we showed that this T cell stimulatory capacity was not due to the presence of B cells in the LAF fraction and could thus only be due to the presence of DC. As a final and definite proof, we compared the T cell stimulatory power of purified BDCA+ lung cells to that of lung macrophages. This really showed that the BDCA+ cells are very strong inducers of T cell proliferation, confirming the assumption that these are true DC.

A very interesting finding is the difference in expression of surface markers involved in antigen presentation and T cell costimulation. Myeloid lung DC express costimulatory molecules (CD40, CD80, CD86, CD54) at higher levels than plasmacytoid lung DC. The latter do express MHCII (involved in antigen presentation and present on all DCs), but at lower levels than MDC. This difference in maturation status might reflect different roles for the various lung DC subsets. One could argue that immature pDC in lung are rather involved in tolerogenic immune responses, while the more mature MDC could preferably act as initiators of an active adaptive pulmonary immune response. It is beyond any doubt that, to justify this hypothesis, much more data (such as analysis of cytokine secretion patterns and evaluation of T cell stimulatory capacity of the different respiratory DC subtypes) are needed than the mere observation that lung pDC have immature surface phenotypes. However, there is some evidence from recently published data that sup-

ports the hypothesis of a tolerogenic role for plasmacytoid DC, at least in animal models (22, 23). Moreover, De Heer and coworkers showed that lung plasmacytoid DCs provide protection against inflammatory responses to harmless antigen in a mouse asthma model (24). It remains to be elucidated if this tolerogenic function of plasmacytoid DC in mice is indeed shared by their human counterparts, and our findings on human lung plasmacytoid DC urge the need for further investigations into this largely unknown population. In addition, the function of the more mature MDC in human lung needs to be explored and the question on the possible differences between MDC1 and MDC2 has to be addressed.

There have been some conflicting data in the literature concerning the presence of CD1a⁺ DC in human lung. We consistently identified CD1a⁺ DC in human lung digests by flow cytometry and in lung tissue sections by immunohistochemistry, demonstrating that their presence was confined to the epithelia of the conducting airways. No CD1a⁺ DC were present in the lung parenchyma. It has been shown that CD1a and CD1c are both present on Langerhans cells in human skin and actually represent one homogeneous population (25). Extrapolation of these findings to human lung would mean that CD1c⁺ (BDCA1) DC in human lung would be identical to the CD1a⁺ Langerin⁺ epithelial lung DC. We found that although there are indeed several DC that coexpress CD1a and CD1c, this is not one homogeneous group, and CD1a⁺ CD1c[−] as well as CD1a[−] CD1c⁺ DC are present in human lung.

These findings are in line with previous studies (19) that used double stainings for CD1a and CD1c on human lung tissue and also demonstrated the presence of CD1a⁺ CD1c[−] DC as well as CD1a[−] CD1c⁺ DC in conducting airways, where the former could mainly be found in the epithelium and the latter in the submucosa. DC that expressed CD1a as well as CD1c were observed in both locations. So, whereas these populations are very closely related, there are some differences between them. One possibility would be that the phenotype of CD1a⁺ DC changes during migration from the epithelium to the submucosa and that this migration is associated with the loss of CD1a and the acquirement of CD1c. Or maybe it is the other way around: CD1c⁺ DC recruited from the blood circulation could lose CD1c and acquire CD1a when they migrate from submucosal tissue to the epithelium. These are only speculations on the possible differences between these cell types, but questions on their function should be addressed in separate studies, including *in vitro* experiments on recruitment and migration of DC and how these movements influence the phenotypical and functional characteristics of DCs. This is not an academic discussion but is of great clinical relevance, because it has been shown in asthma models in mice that allergen exposure to the lung results in a strongly enhanced influx of DC to the lung, followed by migration of allergen loaded DC to the draining lymph nodes, where allergen specific T cell stimulation occurs (26). Moreover, one group described a dramatic increase in CD1c⁺ HLA-DR⁺ cells in the lamina propria of conducting airways of patients with allergic asthma after allergen challenge (21). Although there is no functional analysis of these cells and a nonatopic control group could not be included, the authors conclude that these cells are DCs and suggest an important role for DC in human airway allergy.

In conclusion, we describe the presence of three previously unidentified DC subsets in human lung digests: myeloid DC type 1 (BDCA1⁺/HLA-DR⁺), myeloid DC type 2 (BDCA3⁺/HLA-DR⁺), and plasmacytoid DC (BDCA2⁺/CD123⁺). The presence of CD1a⁺ DC in the epithelium of the conducting airways is confirmed. It is clear that the recruitment and migration of respiratory DC is a possible therapeutic target. The identification and characterization of respiratory DC subsets provides a

more detailed view on the presence of these strong APC in human lung, which is absolutely necessary to further unravel their role in respiratory diseases.

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