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IDENTIFICATION, RARE-EVENT DETECTION AND ANALYSIS OF DENDRITIC CELL SUBSETS IN BRONCHO-ALVEOLAR LAVAGE FLUID AND PERIPHERAL BLOOD BY FLOW CYTOMETRY

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1. ABSTRACT

Dendritic cells (DCs) and their precursors play important roles, not only as antigen presenting cells, but components of the immunoregulatory network. Depending on their lineage, activation and differentiation state, DCs can promote a strong T-cell response or a state of anergy, and can polarize the T helper response in the direction of TH1 or TH2. The lung comprises one of the major interfaces between the host and the environment and regularly comes into contact with antigens, allergens and pathogens. Inflammation in response to these stimuli must be very carefully regulated in the lung since perturbations, especially of a chronic nature, can result in immunopathology that interferes with the lungs' critical function of gas exchange. Especially in disease states such as chronic obstructive pulmonary disease, which has been associated with a chronic TH1 response, and asthma, which is TH2-driven, the ability to access and characterize DCs and their precursors is critical to the understanding of immune modulation in these processes. In this report we will demonstrate that mature DCs and their monocytoid and plasmacytoid precursors can be sampled in the lung by the minimal invasive procedure of broncho-alveolar lavage (BAL) despite their relative scarcity and can be detected by rare event multiparameter flow cytometry.

2. INTRODUCTION

Dendritic Cells and Their Subsets. Dendritic cells constitute a heterogeneous population of potentially powerful inducers and regulators of immune reactivity (1,2). They specialize in the acquisition, processing and presentation of antigens (Ag) to induce major histocompatibility complex (MHC)-restricted T cell-mediated immune responses or tolerance (3,4) and are important in the generation of long-term T-cell memory (5). In addition to DC derived from hematopoietic progenitors, follicular DC, of mesenchymal origin, play an important role in antigen presentation to B cells (6). Hematopoietic DC originate from precursors of both myeloid and lymphoid lineages that circulate in the blood and typically lack cell surface markers for T, B, natural killer, or monocyte/macrophage cell lineages (7). These precursors give rise to immature DC, which localize strategically in non-lymphoid tissues, where they capture and process antigens from invading pathogens. Encounter with antigen, or apoptotic bodies from normal or altered self, triggers the processes of migration to the lymphoid tissues and maturation. The capacity of these APC to activate or tolerize T lymphocytes appears to be related to their location, their lineage (monocytoid or plasmacytoid) and to their state of differentiation/maturation. Distinct DC subsets can differentially regulate T helper (H) cell responses, *in vivo* and *in vitro* (8). Much information has been accumulated from the study of DC matured from blood monocytes (9) or hematopoietic progenitor cells (10) *in vitro*, whereas the study of DC *in vivo* has been hampered because of their rarity. Lymphoid-related DC have a plasmacytoid morphology and are presumed to share a common precursor with T cells (11). Plasmacytoid DC precursors (pre-PDC) are $CD4^+$, $CD123^+$ (IL-3R alpha), $CD11c^-$ and are found in secondary lymphoid organs (12) bone marrow and blood (7). These have also been termed pre-PDC in the literature. They are distinguished from myeloid DC precursors (pre-MDC, or pre-MDC) or mature T cells by unique morphology and surface expression of CD123 (IL-3R alpha chain, (13). By contrast, pre-MDC are $CD4^+$ IL-3R $alpha^-$ $CD11c^+$, and have been shown to produce significant levels of IL-12 (8). Both DC subsets are effective in stimulating $CD4^+$ and $CD8^+$ T cell responses.

DCs and immune regulation in the lung. The lung is a major interface between the host and the environment. As such it comes into contact with inhaled

antigens, allergens and pathogens. Antigen presenting cells in the lung must process and transport antigen to the pulmonary lymphatics where naïve T cells can be stimulated in response to newly encountered antigens, and memory T cells can be activated by recall antigens. All inflammatory responses in the lung must be closely regulated because local effector responses that are too vigorous or too polarized can lead to pathology. For example, chronic obstructive pulmonary disease is associated with chronic TH1 responses (14), whereas asthma has been associated with TH2 responses (15). DC subsets are an early checkpoint in the transition from innate to adaptive immunity and can affect the direction and magnitude of T-cell responses in the lung (16). Generally, MDC polarize in the direction of TH1 and PDC in the direction of TH2 (8). However, the existence of preexisting inflammation, with attendant cytokines and soluble mediators, also greatly influences polarization of the T-cell response (17). Since DC play such a key role in regulating immune it is of great interest to be able to detect and quantify DC subsets in the lung.

Because pre-DC are present in the circulation at low frequency they have been difficult to study. However the identification of DC subsets in peripheral blood and various tissues may be crucial to understanding their immunologic significance. Further monitoring and characterization of these cells may provide insights into their role in wide variety of physiologic and disease processes that involve polarization of the immune response or induction or breach of tolerance. These include HIV infection, organ transplantation, immune reconstitution after hematopoietic stem cell transplantation, graft versus host disease, cancer vaccines, autoimmune disease and asthma, to name a few.

In this study, we have employed rare-event, multi-color, flow cytometric analysis to confirm the presence of discrete populations of rare DC subsets in the lung and in the circulation of normal healthy volunteers. These same strategies can be applied to high speed fluorescence activated cell sorting. Alternatively, lineage depletion can be applied as the first step in sequential immunomagnetic bead separation. This in turn, enables molecular and functional analysis of these rare DC subsets.

3. MATERIALS AND METHODS

3.1. Human subjects

Peripheral blood (5-10 ml of venous blood, Sodium heparin, 10 units/ml, Upjohn, Kalamazoo, MI) was obtained from normal healthy volunteers. We assayed BAL and peripheral blood mononuclear cells (PBMC) obtained from 23 healthy subjects (PBMC=23, BAL=5). Informed consent was obtained from all subjects according to protocols approved by the University of Pittsburgh Institutional Review Board.

3.2. Peripheral blood mononuclear cell (PBMC) preparation

Ten milliliters of heparinized blood from each normal healthy volunteer were diluted 1:2 with Dulbecco's Ca_2^+ Mg_2^+ -free phosphate-buffered saline (PBS-A). PBMC were obtained by centrifugation over Ficoll-Hypaque gradients (Sigma Diagnostics, Inc., Saint. Louis MO) according to the manufacturer's instructions. Harvested cells were washed 3 times with 40ml of PBS-A prior to surface staining. The cell pellet was resuspended in a 15 ml polypropylene conical tube (Falcon, Becton-Dickinson, Franklin Lakes, NJ) in 3 ml staining buffer (PBS-A containing 4% v/v fetal calf serum and 0.1% w/v NaN_3).

3.3. Broncho-alveolar lavage cell preparation

After minimal sedation with midazolam and fentanyl given intravenously, and careful topical anesthesia with 5 ml of 1% lidocaine solution injected through the vocal cords, an Olympus fiberoptic bronchoscope was passed transnasally, advanced to a subsegment of a middle lobe, and wedged. Bronchoalveolar lavage was conducted by instilling 2 aliquots of 100-150 ml of sterile, 37oC 0.9% NaCl with recovery by wall suction. Returned fluid was kept on ice until processing. Fluid was pooled, filtered though cotton gauze under vacuum, centrifuged at 400xg for 10 minutes, and resuspended in 3 ml staining buffer.

3.4. Staining and flow cytometric analysis of PBMC and BAL cells

BAL cells were discarded material obtained for diagnostic purposes. Approximately 2 to 4 million cells were stained for flow cytometry. Cells were distributed to 3 tubes (1.25 ml Eppendorf polypropylene), centrifuged at 400 x g for 5 min at 4^{o}C and decanted to a dry pellet. Nonspecific loss of DC during sample preparation due to adherence was minimized by the use of polypropylene tubes and by holding samples at 4^{o}C prior to fixation. The pellet was first incubated for 20 min on ice with 2 microliters each of FITC-labeled lineage antibodies (Abs) directed against CD3 (Beckman-Coulter 1281), CD14 (Beckman-Coulter 0645), CD19 (Beckman-Coulter 1284), CD33 (Beckman-Coulter 1135), and CD57 (Beckman-Coulter 0466). The cells were centrifuged (400 x g for 5 min at 4^{o}C), and the dry pellet was incubated on melting ice for 20 min with 2 microliters each Ab conjugated to PE, ECD and PE-Cy5 as shown in table 1. After an additional wash, the cells were resuspended in approximately 1 ml of equal volumes of staining buffer and 0.5% methanol-free formaldehyde in PBS. At least 2 x 10^6 events were collected, using a four-color Beckman-Coulter Epics XL cytometer set on low flow rate at a maximum of 1,000 events/sec. Cells from tube 1 (table 1) were acquired in triplicate (total: 6 x 10^6 cells). An acquisition list gate was established on linear forward light scatter (FS) and log side angle light scatter (LSSc) that included all viable cells and excluded sub-cellular debris and event-clusters in the last channels.

4. RESULTS

Flow cytometric acquisition and analysis of PBMC and BAL cells. Flow cytometry data were analyzed in real time using Beckman-Coulter System II software. Data were saved as FCS 2.0 Listmode files for subsequent reanalysis with WinList (Verity Software House, Topsham

Table 1. Monoclonal antibodies used to define pre-MDC, pre-PDC and mature DC

Specificity	FITC	PE	ECD	PE-Cy5
pre-MDC	Lineage [1]	Anti-CD11c (Becton-Dickinson 347637, clone S-HCL-3)	Anti-CD4 (Beckman-Coulter 6604727, clone SFCI12T4D11)	anti-HLA-DR (Beckman-Coulter 2659, clone Immu-357)
pre-MDC and mature DC Isotype control	Lineage [1]	Isotype IgG_1 control (Beckman-Coulter IM0639, clone 679.1Mc7)	Anti-CD4 (Beckman-Coulter 6604727, clone SFCI12T4D11)	anti-HLA-DR (Beckman-Coulter 2659, clone Immu-357)
pre-PDC	Lineage [2]	Anti CD123 (IL-3R alpha) (PharMingen 18765B, clone 7G3)	Anti-CD4 (Beckman-Coulter 6604727, clone SFCI12T4D11)	anti-HLA-DR (Beckman-Coulter 2659, clone Immu-357)
pre-PDC Isotype control	Lineage [2]	Isotype IgG_1 control (Beckman-Coulter IM0639, clone 679.1Mc7)	Anti-CD4 (Beckman-Coulter 6604727, clone SFCI12T4D11)	anti-HLA-DR (Beckman-Coulter 2659, clone Immu-357)
Mature DC	Lineage [1]	Anti-CD83 (Beckman-Coulter IM2218, clone HB15a)	Anti-CD4 (Beckman-Coulter 6604727, clone SFCI12T4D11)	anti-HLA-DR (Beckman-Coulter 2659, clone Immu-357)

[1] For pre-MDC and mature DC the lineage cocktail consisted of antibodies to CD3 (Beckman-Coulter 1281, clone UCHT1), CD14 (Beckman-Coulter 0645, clone RMO52), CD19 (Beckman-Coulter 1284, clone J4.119), and CD57 (Beckman-Coulter 0466, clone NC1). [2]For pre-PDC only, antibodies to CD11c (Caltag MHCD11c01) and CD33 (Beckman-Coulter 1135, D3HL60.251) were added.

ME). Seven parameters were acquired and stored: FS, LSSc, Log FL1-FL4, and time. $CD4^{bright}$ and lineage $marker^{negative}$ events were identified using bivariate plots of anti-CD4 versus LSSc and lineage cocktail versus LSSc, respectively (figure 1 panels A and B). A compound logical gate excluding all other cell populations was applied to a bivariate plot of anti-HLA-DR versus anti-IL-3R alpha. $HLA\text{-}DR^+$, $CD123^+$(IL-3R alpha), $CD4^+$, $lineage^-$ cells with lymphoid light scatter were quantified on this plot (figure 1 panel D). An analogous strategy was applied to the detection of pre-MDC and mature DC subsets. Again, $lineage\ marker^{negative}$ and $CD4^{bright}$ events were identified by series of compound gates. Then, pre-MDC and mature MDC were identified by HLA-DR and either CD11c or CD83, respectively. $HLA\text{-}DR^+$, $CD11c^+$, $CD4^+$, $lineage^-$ cells with lymphoid light scatter were quantified as pre-MDC (figure 1 panel E), $HLA\text{-}DR^+$, $CD83^+$, $CD4^+$, $lineage^-$ cells as mature MDC (figure 1 panel F).

We determined the lower limit of detection of this assay using isotype controls in place of the antibodies identifying pre-MDC and pre-PDC (HLA-DR plus CD11c or CD123 (IL-3R alpha), respectively). Owing to the use of multiple parameters and compound gates, false-positive events were exceedingly rare. In peripheral blood, the lower limits of detection for pre-MDC and pre-PDC were estimated as the 99th percentile of background staining (mean percent positive plus 2.6 standard deviations) and were 0.00027% and 0.00031%, respectively, expressed as a percentage of mononuclear cells. pre-MDC and pre-PDC were identified at low frequency in the blood of control subjects (0.342% and 0.115%, respectively) and were even less prevalent in BAL (0.250% and 0.042%, respectively). Mature DC were below the lower limit of detection among normal PBMC, but comprised 0.077% of normal BAL cells.

5. DISCUSSION

The lung provides a major barrier between the host and the environment. Local immune responses in the lung must protect the host without interfering with its critical function of gas exchange. Although DC are a minor cell population in the lung, their long dendritic processes are estimated to cover 25-30% of the barrier area (16). We detected pre-MDC and pre-PDC at low frequency among both PBMC and BAL cells from healthy subjects. Normal BAL and normal PBMC have similar frequencies of pre-MDC, but the normal lung has fewer pre-PDC than PBMC, perhaps reflecting its role as a site of initiation of mucosal immune responses. We observed mature DC at low frequency in normal BAL, but not PBMC. These were most likely washed from the epithelial surfaces. In contrast to mature DC, which initiate primary immune responses, pre-MDC and pre-PDC are believed to modulate and direct immune responses and, perhaps, play a role in the maintenance of tolerance.

The detection of pDC and DC in BAL samples required a strategy that is common to all "rare event" problems in flow cytometry. The term "rare-event analysis" usually refers to detection of events that occur at a frequency of 1 in 1000 (0.1%) or less, although the current "record" claimed in the literature is 1 in 10,000,000 (0.00001%) for tumor cells spiked into peripheral blood (18). Detecting an event at low frequency requires both a high signal to noise ratio and the acquisition of a large number of events. Technical aspects of rare-event detection have been reviewed by several authors (19-23). What follows is a detailed discussion of this strategy with emphasis on the problem of detecting DC in the lung.

Sample Concentration and Flow Rate. Event frequency is a property of the sample and not something that can be manipulated, save by a pre-enrichment step prior to analysis (24). For example, knowing that dendritic cell precursors, which are rare in the peripheral circulation, do not express lineage markers present on T cells, B cells, NK cells or monocytes (all of which are common), it possible to physically deplete cells bearing these lineage

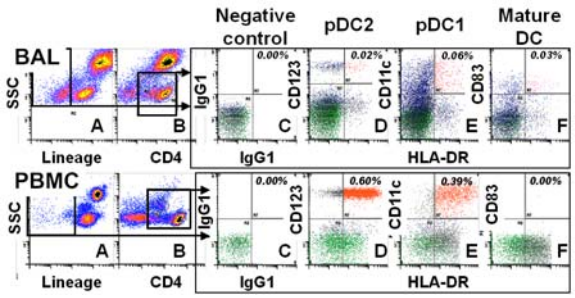

Figure 1. Detection of pre-PDC, pre-MDC and mature DC in BAL and peripheral blood. Panels A and B show the gating strategy used to limit the analysis to lineage negative (A), $CD4^+$ (B) cells. Panel C-F make use of this compound gate. It should be noted that CD11c and CD33 were included in the lineage cocktail used to define pre-PDC but not pre-MDC. Panel C, the isotype controls show no nonspecific events despite denominators of 1.3 million, and 0.7 million, events per tube for BAL and PBMC samples, respectively. Panel D shows HLA-DR^+/$CD123^+$ pre-PDC, which are more prevalent in peripheral blood than in BAL. Panel E shows HLA-DR^+/$CD11c^+$ pre-MDC. Panel F shows HLA-DR^+ $CD83^+$ mature DC, which are undetectable in the peripheral circulation, but present at low frequency in BAL. The percent double positive cells (of total mononuclear cells) is shown in the upper right hand quadrant of each histogram.

positive cells by immunomagnetic separation, effectively enriching the rare population of interest many-fold. The advent of high-speed analysis obviates the need for physical pre-enrichment, although same principle is applied virtually. Cells are stained with a cocktail of linage markers (CD3, CD14, CD19, CD57), all labeled with the same fluorochrome. Cells expressing any of these markers are logically "gated out" of the analysis (7). This technique also has the advantage of preserving the quantitative accuracy of the analysis, which is compromised when a physical pre-enrichment step is performed.

The frequency of the event of interest in the sample is one of the parameters which dictates how many total cells must be processed. Obviously the lower the frequency of the events of interest, the more events it will be necessary to acquire. The rate of sample acquisition on the flow cytometer can be manipulated by concentrating the sample or by increasing the sample flow rate. Although it is tempting to shorten acquisition time by increasing the flow rate, this approach has its limitations. Analytical cytometers have low, medium and high flow rate settings, which correspond to the rate at which sample is drawn into the cytometer. An increase in the flow rate is accomplished by increasing the sample pressure relative to the sheath pressure. This results in a wider sample stream within the sheath fluid stream, increasing the opportunity for two cells to pass through the detection system at the same time (coincidence). It also increases the coefficient of variation (CV) of all of the measured parameters since the position of an individual cell within the stream during interrogation is more variable. A better way to maximize the event rate is to optimize the sample concentration. For example, if your cytometer draws sample at 30 microliters per minute (medium speed on some conventional-speed cytometers), the sample concentration (in cells/mL) divided by 2000 is approximately equally to the sample acquisition rate in cells per second. In this example, concentrating the sample to 6 x 10^6 cells/mL yields a sample acquisition rate of 3000 events per second, a speed that is close to the maximum event rate on all but the highest-speed instruments available today. It should be noted that there is no advantage to concentrating the sample beyond the capacity of the instrument. At higher flow rates it becomes important to gate out doublets using doublet discrimination (comparison of signal pulse height and width or area).

Signal to Noise. Successful detection of rare events depends on maximizing the signal to noise ratio. Noise comes from many sources including the nonspecific binding of a fluorochrome of interest, cellular autofluorescence, disruptions in fluidics, and other electrical or mechanical problems. There are several tricks for maximizing the difference between signal and noise. One very important aspect is to characterize the total noise using an appropriate negative control. Sometimes, it is possible to devise a control sample that is identical to the experimental sample in all respects, except that it does not contain the rare event of interest (25) or, it may be necessary to use control reagents such as isotype-matched fluorochrome-conjugated antibodies (7). Isotype controls may be difficult to interpret and should be similar to the experimental reagent with respect to the ratio of fluorescent dye to protein (F to P ratio), and the antibody concentration used for staining. Although the F to P ratio is not readily available for commercially manufactured products, individual manufacturers attempt to standardize this parameter as a quality control measure. In the real world, sample limitations sometimes preclude the use of vendor specific isotype controls (table 1). Although this does not necessarily render the results uninterpretable, this is not the optimal situation. Additionally, staining with isotype controls must be designed so that the same gating strategy used to detect the rare event can be applied to the negative control sample (lineage negative, CD4 positive). It is important to point out that an identical number of events must be acquired for the negative control sample as for the experimental sample (1-2 million events if frequency of event of interest is ~ 1/100,000). Resist the temptation to acquire fewer events because there is "nothing there" in the negative control. It is also important to note that if the frequency of spuriously positive events is 0.02% (1 in 5000), it is impossible to use this assay to detect most DC subsets present at a frequency of 1 in 10,000, no matter how many events are acquired. Thus, the frequency of false positive events determines the lower limit of detection. It follows that there is a point of diminishing return beyond which acquiring a greater numbers of events will increase the precision of the rare event frequency estimate, but will not increase the sensitivity of the assay.

Two important factors bear on the signal to noise ratio: the difference in fluorescence intensity between negative and positive populations and their variances

(usually expressed as a coefficient of variation, or ratio of the standard deviation and the mean fluorescence intensity). Some membrane or cell-associated dyes give very bright signals and therefore place cells far from noise. When using combinations of fluorochrome-conjugated antibodies, we often reserve PE for the most critical measurement (CD11c, CD123, CD83), because the absorption and emissions spectra are widely separated, and the extinction coefficient and quantum yield are high compared to other fluorochromes (26)

When autofluorescent myeloid cells, cellular debris or red blood cells interfere with rare event detection it is often possible to move them out of the way by targeting them with a specific antibody (e.g. CD14 for monocytes/macrophages). We have used this strategy to identify DC subsets that comprise a small proportion (1 in 10,000 to 1 in 100,000) of bronchoalveolar lavage cells (27).

Another important aspect of the overall signal to noise is the number of parameters used to define the rare event of interest. Modern multi-laser, multi-PMT instruments permit detection strategies that make use of multiple parameters to define the rare event. As described above, the rare event of interest should be positively identified by more than one fluorescence parameter (CD4+ HLA-DR+ and CD123+). Not quite so obvious is the importance of including at least one fluorescence parameter for which the rare event is negative (in the case of DC subsets, these cells are lineage negative). In rare event detection, it is almost as important to specify where the rare event is NOT, as to specify where it is. This is especially helpful for defining a set of compound logical gates that assign the population of interest a unique location in multiparameter space. Compound gating strategies that maximize detection of events in the positive control while minimizing false positive events in the negative control can be determined empirically and applied to the experimental data. Such analyses are performed after the fact, on listmode datafiles. Using these principles we were able to detect pre-PDC subset in a BAL of a lung transplant recipient at a frequency of 1 cell per 100,000 (28).

Cellular autofluorescence can interfere greatly with rare event detection by increasing noise. It is due primarily to the presence of native fluorescent intracellular molecules such as flavins (29), which are excited by blue-green light (the 488 line provided by Argon lasers) and emit over a broad range of wavelengths. As long as the rare event of interest is not itself highly autofluorescent, autofluorescent cells can be eliminated from the analysis by acquiring an unstained fluorescence parameter or by staining with an antibody that is known not to react with the rare event of interest (lineage positive cells). Cells positive for this parameter can be logically eliminated during analysis with a "Not gate."

The time parameter, which can be saved as a listmode parameter or calculated offline, can also help one recognize and eliminate episodic noise encountered during long sample acquisitions. Plotting a single parameter (e.g. forward scatter) as a function of time facilitates the identification of event bursts caused by minor clogs or other transient problems. Another familiar problem that can be eliminated using the time parameter is the flurry of spurious events that may occur if a sample tube is allowed to run dry and air is aspirated. These deviations can be recognized and removed from the analysis with a logical gate.

Reproducibility**.** Anyone who has ever looked at the result of a successful rare event experiment knows that the results are often unimpressive. There, against a denominator of a million or more acquired events are a small number of positive events that have been filtered through a series of gates defining the population of interest. Sometimes the events form a tight cluster in a 2-parameter scatterplot (e.g CD123 vs HLA DR). Sometimes they appear to be more diffuse in 2-parameter space but are unique in multiparameter space (e.g. CD11c vs HLA DR together with "tight" CD4 vs logSSC). The credibility of such determinations can be greatly enhanced by performing replicate determinations. During assay development, we routinely stain 3 independent samples, acquiring the same number of events for each. Thus the frequency of rare events can be reported as the mean and the associated confidence interval and a coefficient of variation (ratio of the standard deviation to the mean) can be calculated. For example, in the present studies pre-PDC were determined in triplicate for 3 subjects. The intra-subject coefficient of variation was 6.9%, indicating high reproducibility despite the relative rarity of the event being measured. The frequency of false positive events detected in the negative control sample (also collected in replicate with the same number of events as the experimental sample) should also be reported, and a lower limit of detection calculated as the upper 99th percentile of negative control. In our DC studies the lower limits of detection were 0.00027% and 0.00031% (< 1/300,000), respectively (7), whereas in our MHC tetramer studies it was only 1/8,000 (30). This illustrates the importance of the negative control for the interpretation of rare event data.

Taken together, this example demonstrates how carefully designed multi-parameter flow cytometric experiments can yield quantitative data on rare populations of cells as they exist *in vivo*. The availability of a new generation of flow cytometers capable of greater than 4 colors opens the way for functional studies on rare populations in minimally manipulated samples.

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Abbreviations: Ab, antibody;Ag, antigen;APC, antigen-presenting cell;BM, bone marrow;BAL, broncho-alveolar lavage;CD, cluster of differentiation;DC, dendritic cell;ECD, energy coupled dye;FITC, fluorescein isothiocyanate MHC, major histocompatibility complex;PE, phycoerythrinPE-Cy5, PE-cyanine 5PBMC, peripheral blood mononuclear cells

Key Words: Dendritic Cell, Rare Event Detection, Flow Cytometry, Broncho-alveolar Lavage, Plasmacytoid, Monocytoid, Lung

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