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Flow cytometry of sputum: assessing inflammation and immune response elements in the bronchial airways

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Abstract

Background—The evaluation of sputum leukocytes by flow cytometry is an opportunity to assess characteristics of cells residing in the central airways, yet it is hampered by certain inherent properties of sputum including mucus and large amounts of contaminating cells and debris.

Objective—To develop a gating strategy based on specific antibody panels in combination with light scatter properties for flow cytometric evaluation of sputum cells.

Methods—Healthy and mild asthmatic volunteers underwent sputum induction. Manually selected mucus “plug” material was treated with dithiothrietol, filtered and total leukocytes acquired. Multicolor flow cytometry was performed using specific gating strategies based on light scatter properties, differential expression of CD45 and cell lineage markers to discriminate leukocytes from squamous epithelial cells and debris.

Results—The combination of forward scatter and CD45 expression reliably segregated sputum leukocytes from contaminating squamous epithelial cells and debris. Overlap of major leukocyte populations (neutrophils, macrophages/monocytes) required the use of specific antibodies (e.g. CD16, CD64, CD14, HLA-DR) that differentiated granulocytes from monocytes and macrophages. These gating strategies allowed identification of small populations of eosinophils, CD11c+ myeloid dendritic cells, B cells and NK cells.

Conclusions—Multicolor flow cytometry can be successfully applied to sputum samples to identify and characterize leukocyte populations residing on the surfaces of the central airways.

Keywords

induced sputum; flow cytometry; immunophenotype; methods; human

INTRODUCTION

Flow cytometry (FCM) is a useful tool for a variety of both clinical diagnostic and research applications and is used extensively for immunophenotyping blood. The rationale for its

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DECLARATION OF INTERESTS

The authors report no declarations of competing interest.

application to sputum is based on the fact that induced sputum techniques provide a mechanism for procuring viable cells derived from surfaces of central airways (Alexis et al. 2001b), a region of the lung which is difficult to sample non-invasively in humans. While sputum induction retrieves sufficient cells for FCM, its application to sputum samples remains relatively novel, despite its potential for yielding valuable information on the functional and phenotypic characteristics of inflammatory and immune cells residing on bronchial surfaces.

Unlike blood and bronchoalveolar lavage (BAL) samples, sputum contains variable amounts of mucus, contaminating epithelial cells and endogenous or exogenous debris which can render FCM analysis challenging, likely causing reluctance by many investigators to use it for evaluating sputum. As a result, BAL cells are often used as surrogates for cells residing in the central airways; however, this is less than optimal because these techniques sample different regions of the lung (central airways vs. alveolar parenchyma) and the leukocyte composition (Table 1) is quite different between the two. (Balbi et al. 2007; Bienkowska-Haba et al. 2002; Pizzichini et al. 1998; Grootendorst et al. 1997; Alexis et al. 2000) BAL and sputum cells also possess different functional and phenotypic characteristics. (Alexis et al. 2000) For these reasons, application of FCM to sputum samples (as opposed to BAL) is, in our view, preferable and, perhaps, more appropriate for assessing inflammation of the bronchial airways.

Early studies, (Alexis et al. 2001a; Alexis et al. 2000) as well as some more recent FCM studies from other labs were somewhat technically limited in that they relied primarily on light scatter properties for gating sputum populations and used only two fluorochromes (FITC and PE) for analysis of sputum. Thus anti-CD45, a critical marker for differentiating leukocytes from debris was generally not used. As a result, the purity of gated leukocyte populations in these studies likely suffered. Some studies have used specific cell lineage markers (e.g.: CD3, CD4, CD14, HLA-DR or other markers) to identify and gate specific cells of interest (Barry et al. 2002; Barry and Janossy2004; Frankenberger et al. 2004; Janossy et al. 2008; Holden et al. 2008; McCarthy et al. 2007) and a few more recent studies (Leckie et al. 2003; Dominguez et al. 2004; Jaksztat et al. 2004; Lay et al. 2007; Alexis et al. 2009; Antoniou et al. 2005; Dua et al. 2010) employed more intricate approaches; however, none have provided detailed descriptions of a comprehensive and rigorous approach using FCM for differentiating sputum leukocytes from debris.

Here we describe strategies using a combination of light scatter properties, CD45 expression, and differential expression of lineage-specific cell surface markers which allow more confident identification of sputum leukocytes. Antibody combinations were designed to assess expression of specific cell surface proteins associated with innate and adaptive immunity. Surface proteins associated with innate immune function include Fc-gamma receptors (FcγRI/CD64 and FcγRIII/CD16), the LPS receptor (CD14) and complement receptors (CR3/CD11b; CR4/CD11c), as well as Toll-like-receptors 2 and 4 (CD282 and CD284, respectively) and the low- and high-affinity IgE receptors (CD23/FcεR-IIα and FcεRIα, respectively). These are found primarily on mononuclear phagocytes and granulocytes and to lesser degree on other leukocytes. While they function primarily in the innate immune system, their functions also overlap with adaptive immune responses. Proteins associated primarily with adaptive immune responses include HLA-DR/MHC-II, co-stimulatory molecules CD80/B7.1, CD86/B7.2, CD40 and CD83 (tubes 4, 5 and 6), which are expressed primarily on antigen presenting cells (APC) including DCs, monocytes, macrophages and lymphocytes. They function in antigen presentation and activation of T-cells and B-cells to elicit antigen specific immune responses and modulate immune-mediated inflammation.

The methods described here provide detailed guidance to investigators in both research and clinical settings who wish to use FCM for evaluating sputum samples.

METHODS

Extended descriptions and discussion of methods presented here are included in the Appendix.

Subjects and IRB approval

Induced sputum was obtained from both healthy and mild allergic asthmatic or allergic non-asthmatic volunteers under our screening protocol which was reviewed and approved by the University of North Carolina Committee on the Rights of Human Subjects (Institutional Review Board). Asthmatic volunteers had mild symptoms of asthma as defined under section 3 of the 2007 NHLBI guidelines for the diagnosis and management of asthma and all had a positive methacholine challenge test ($PC_{20} \leq 10$ mg/ml), defined as a 20% decrease from the baseline forced expiratory volume in the first second (FEV_1) following an inhaled provocative challenge dose of ≤ 10mg methacholine/ml. Atopy was demonstrated by a positive immediate skin test response to one of the following allergen mixes: 2 species of house dust mite (Dermatophagoides farinaea and Dermatophagoides pteronyssinus), cockroach, tree mix, grassmix, weedmix, moldmix1, moldmix2, rat, mouse, guinea pig, rabbit, cat or dog.

Sample Collection and Processing

Sputum induction and processing techniques are based on the those of Hargreave et al (Hargreave et al. 1998) and are detailed elsewhere. (Alexis et al. 2000) Briefly, only the cell-enriched mucus “plugs” manually selected from the surrounding clear fluid were processed to minimize squamous cell contamination and dilution from saliva. (Pizzichini et al. 1996; Spanevello et al. 1998; Alexis et al. 2006) Sputum samples should immediately be placed on ice and processed within 2 hours.(Efthimiadis et al. 2002a) Excessive or prolonged exposure to dithiothrietol (DTT) during processing adversely affects both cell viability and surface marker expression (Efthimiadis et al. 1997; Loppow et al. 2000) and should be avoided. (See the appendix for additional discussion).

Antibody Panels and Staining Procedures

“Standard” antibody panels (Table 2) were used for identifying leukocyte populations and assessing expression of select cell surface proteins associated with innate and adaptive immunity, antigen presentation and inflammation. Anti-CD45 (leukocyte common antigen) was included in all sample tubes to help differentiate leukocytes from debris and assist in identifying leukocyte populations. Major lineage markers such as CD16, CD14, HLA-DR, CD3, CD19 and CD56 were used to aid in isolating specific populations. Certain antibodies were included in multiple sample tubes to facilitate gating of specific populations. Incorporation of additional fluorochromes would be advantageous, allowing consolidation of tubes and conservation of cells. Though we have done this in some panels for specific protocols, it is often difficult to procure specific antibodies labeled with the desired fluorochrome.

Specific staining procedures have been described elsewhere (Alexis et al. 2000) and in the Appendix. Following staining, cells were fixed in 1% paraformaldehyde in Dulbecco’s phosphate-buffered saline (DPPS) and FCM performed within 24 hours. Stained samples should be refrigerated at 4°C until acquired. Sources for specific antibodies are listed in the appendix (Table 4). Specific antibodies were titrated to determine an appropriate amount for staining. We found it necessary to titrate and dilute isotype controls up to 1:5 (or more),

especially for IgG2 isotypes since non-specific binding resulted in fluorescent intensity of the isotype control exceeding that of certain specific antibodies. Though fluorescence minus one (FMO) (Tung et al. 2004) controls would be preferable to isotype controls for determining background fluorescence, it was not feasible since it would require an excessive amount of cells to be performed properly. (See the appendix for additional discussion of isotype controls.)

Determination of Background Fluorescence Using Isotype Controls

Mean fluorescence intensity (MFI) of isotype controls (Tubes 1 and 2, Table 2) for specific populations was subtracted from the MFI measured for specific markers to control for background auto-fluorescence and non-specific fluorescence. For individual populations, background fluorescence was determined simultaneously for all fluorescence channels by creating a single histogram and gating 99% of the population (95% at the minimum) in only one fluorochrome channel, usually FITC. As illustrated for the monocyte population (Table 3), background MFIs for the various fluorochromes were relatively constant regardless of which channel was used for gating. This is also true for the other leukocyte populations and avoids the creation of a very large number of histogram gates.

Instrument Setup

We used a BD™ LSR-II digital flow cytometer equipped with 405nm solid state, 488nm argon-ion, and 633nm Helium-Neon lasers, appropriate filters and capability for cross-laser compensation (BD Biosciences Immunocytometry Systems, San Jose, CA). Our “standard” panel uses 5 colors exciting off the 488 and 633 lasers; however, additional fluorochromes, including those exciting off the 405 laser, are used in some studies. Compensations for spillover and spectral overlap were established for a particular set of instrument settings using BD™ CompBeads (BD Biosciences, cat 552843) and an automated compensation algorithm in BD™ FACSDiva 6.1 software (BD Biosciences).

The high auto-fluorescence of sputum macrophages, relative to lymphocytes, requires a compromise to allow application of a single set of PMT voltages for all populations. Our approach has been to optimize instrument settings for monocytes. (see Appendix)

STATISTICS

Statistical evaluation was performed using GraphPad Prism 5.03 software. Non-parametric statistics (Wilcoxon Signed Rank test or Mann-Whitney test) were used to compare groups. Spearman’s correlation was used to test correlation. Least-squares goodness of fit was used to calculate linear regression.

RESULTS

Identification of leukocytes in sputum samples

Figures 1 through 5 represent typical moderately contaminated sputum samples from healthy and mild asthmatic volunteer subjects and demonstrate a typical level of contaminating debris, moderate to high proportion of squamous epithelial cells and small proportion of EOS.

The levels of contamination in dot plots of a relatively uncontaminated and a more typical moderately “dirty” (i.e. contaminated) sputum sample are demonstrated in Figure 1A and B, respectively. Due to the broad range of cell size and granularity, forward and side scatter (FSC and SSC, respectively) was best displayed using a log scale.

In the majority of samples, leukocytes could not be reliably identified using light scatter properties alone, because they are intermingled with contaminating epithelial cells, detritus from dead cells, yeast, bacteria, micro-particles and exogenous inhaled particles. It was therefore essential to include anti-CD45 (leukocyte common antigen) in the antibody cocktail to facilitate leukocyte identification. Even though "leukocytes," expressing CD45 could be segregated from the whole sample (Fig 1C), it is clear that, based on FSC (Fig 1D), a large proportion of the CD45(+) material is much smaller (lower FSC) than expected for leukocytes. We postulated that this is likely due to specific or non-specific CD45 staining of fragmented cell membranes and cell detritus, or diffusion of labeled antibody through leaky membranes of apoptotic, pyknotic or degenerate cells. This is supported by the fact that the majority of this CD45(+)/FSC_{low} material stains positive with propidium iodide (PI), a cell-impermeant stain for viability, as demonstrated in Fig1E. To address this artifact, it was essential to exclude this material based on FSC (Fig1F, High FSC gate), especially for very "dirty" samples. All subsequent gating was done using the "cleaned" CD45 (+) cells (Fig 1F).

Leukocyte populations are similarly positioned in both blood and sputum, based both on light scatter properties and CD45 expression. Neutrophils (polymorphonuclear cells, PMNs) are usually abundant in sputum samples even in healthy individuals and, as in blood, lymphocytes are the smallest and least granular cells in sputum. PMN and, when present, lymphocyte populations served as convenient "landmarks" for locating sputum leukocytes by FCM. The use of density plots (as opposed to dot plots) can facilitate identification of PMN in sputum samples Fig 1C – H). The simplest gating strategy for leukocyte sub-populations was based on SSC and differential CD45 expression (Fig 1G). However, as demonstrated in the following sections, more complex strategies were required to adequately discriminate overlapping populations and subpopulations, especially small or rare-event populations (e.g. EOS, DCs, and basophils) and detection of dimly expressed surface proteins.

When present in sufficient number, squamous epithelial cells appeared as a separate population with high forward and side scatter (Fig 1B & C) and might easily be mistaken for macrophages. However, in contrast to macrophages, this population does not express CD206 (macrophage mannose receptor, Fig 1H) or HLA-DR (not shown) and was absent from samples having minimal squamous cells based on examination of stained slides (not shown).

Specific Gating Strategies

We have found that analysis was facilitated if lymphocyte and granulocyte populations were identified and gated first. Lymphocytes could be identified based on SSC and CD45 expression alone (Fig 2A). Granulocytes were identified within the non-lymphocyte gate (P2) using specific markers (e.g., CD64, CD16, CD14 or HLA-DR) which allowed for their discrimination either through positive or negative selection. Remaining non-granulocytes comprised monocyte, macrophage and DC populations, which were further differentiated based on differential SSC properties, CD45 expression and specific surface markers. Depending upon the antibody panel, it was not always possible to differentiate small populations such as DCs or EOS; however, inclusion of additional population-specific antibodies could facilitate their identification (see below).

Though differential expression of so called "lineage-specific" markers greatly facilitates discrimination of leukocyte populations, some overlap in expression of these molecules on various populations does occur (e.g. CD23 and CD14 on activated PMNs) and may result in less than optimal separation. As a result, gating necessarily becomes relatively more subjective and may vary depending upon the bias of individual analysts. Though this is largely unavoidable, subjectivity and misidentification of populations can be minimized by

gating strategies based on two differentially-expressed markers or sequential "Boolean" gating strategies using two or more markers. The use of relatively conservative gating (i.e. gating tightly around populations) in such situations will also help to minimize misidentification of populations.

Evaluation of Innate Immune Response Proteins (Table 2, Tubes 3, 7 & 8)

Lymphocytes could be gated confidently based on SSC and CD45 expression (Fig 2A): however, antibodies against specific cell surface proteins were utilized to differentiate overlapping populations of PMNs, EOS and mononuclear phagocytes. Differential expression of CD64 and CD16 (tube 3) permitted isolation of PMNs, EOS and DCs from remaining monocytes and macrophages (Fig 2B). Neutrophils and EOS were differentiated based on CD16 expression (Fig 2 C). Keep in mind that degranulated EOS will have lower SSC properties and may blend with the PMNs based on SSC. The remaining non-granulocytes (Fig 2C) were monocytes, macrophages and DCs, which were recombined with the "not-P3"population (Fig 2B) and further partitioned on the basis of light scatter and differential expression of CD45, CD16 and CD14 (Fig 2D & E). Dendritic cells (Fig 2E) were identified as $CD14_{dim}/CD16_{dim}$ and fell primarily within the monocyte population. Granulocytes in tube 7 (Fig 2F) were identified as $HLA\text{-}DR_{dim}CD14_{dim/neg}$ cells and DCs could be identified as $CD14_{dim//}HLA\text{-}DR_{high}$ non-lymphocytic cells (Fig 2G). The proportions of DCs identified in tubes 3 and 7correlated well with the more definitive identification of DCs and their identity can be verified using the lineage cocktail (not shown) as in tubes 4 and 5 (described below).

The expression of CD14 on activated PMNs (tube 8) often prevented good separation of PMNs from mononuclear phagocytes based solely upon CD14 expression. Differential expression of CD14 in combination with either CD23 or FcεRIα (dim expression on granulocytes) was used to facilitate isolation of granulocytes in this tube (Fig 2H). Though some activated PMNs and EOS may express low to moderate levels of these surface proteins, they are expressed at relatively low levels compared to mononuclear phagocytes. Inclusion of anti-HLA-DR would allow better discrimination of mononuclear phagocytes and identification of DCs.

Evaluation of Adaptive Immune Response Proteins (Table 2, Tubes 4, 5 and 6)

Dendritic cells were specifically identified (tubes 4 and 5) within the entire CD45(+) leukocyte population as $Lineage_{dim/negative}/HLA\text{-}DR_{high}$ cells (Fig 3A). The lineage cocktail (Table 2) allowed differentiation of DCs from monocytes, macrophages, granulocytes, T-cells, B-cells and NK cells. Similar to blood, sputum DCs comprised a very small proportion (0.5 – 1.5%) of total sputum leukocytes and were dispersed mainly within the monocyte population (Fig 3B). The majority (≈ 90 to 95%) of sputum DCs were CD11c (+) myeloid DCs (Fig 3C), expressing high levels of CD86. Around 50% or more constitutively expressed CD83 while only a small proportion expressed CD1a (Fig 3D), suggesting that airway luminal DCs are relatively mature and at least partially activated. Subsets of DCs (Fig 3E) expressed both TLR2 and TLR4 (tube 7).

Granulocytes were differentiated within the remaining (non-DC) cells based on a combination of SSC properties, differential expression of lineage markers and the negative or dim expression of either HLA-DR or co-stimulatory molecule CD86 (Fig 3F) in tube 4 or CD83 in tube 5. EOS were differentiated from PMNs as lineage-dim granulocytes (Fig 3F), similar to Fig 2C. Though granulocytes can usually be differentiated from mononuclear phagocytes on the basis of HLA-DR alone (Fig 3G), the use of a combination of two differentially expressed markers is preferable when possible. In most cases, the separation of granulocytes was well-defined; however, some PMNs and EOS may express low levels of

HLA-DR and/or co-stimulatory molecules (CD80 and CD86), sometimes making the distinction between the populations less discrete. Mononuclear phagocytes were further dissected via SSC and differential expression of CD45 (see Fig 2D).

Differential expression of CD206 and HLA-DR (tube 6) allowed discrimination of granulocytes from macrophages (see Fig 1H). We found that CD80 was occasionally expressed at moderate levels on a small proportion of sputum granulocytes (Fig 3H). Though HLA-DR (+) lymphocytes are, for the most part, CD40 (+) B-cells (see Fig 4D) and also express some level of CD86, some activated T-cells may also express these proteins. Inclusion of additional antibodies to specifically identify B cells (tubes 4 and 5) and either lineage cocktail or CD14 to allow identification of DCs (tube 6) would be advantageous.

Sputum Lymphocytes

Lymphocytes (tubes 9 and 10, Table 2) comprise a small and quite variable proportion of sputum leukocytes which is influenced by various factors including the health status of the individual and inflammatory processes in the bronchial airways. In this cohort of healthy,mild asthmatic and atopic individuals, the majority (≈ 80 to 90%) of sputum lymphocytes were CD3 (+) T cells, predominantly (≈ 60 to 65%) CD4 (+) T-helper cells (Fig 4A). B cells and natural killer (NK) cells comprised small and highly variable proportions (Fig 4B) of the lymphocytes. The majority of CD16/56 (+) NK cells were CD3 (+) NKT cells (Fig 4C). B cells (tube 6) were identified as HLA-DR_{high}/CD40 $_{high}$ lymphocytes (Fig 4D). In the absence of CD56, NK cells could be tentatively identified as $CD14_{dim}$/$CD11b_{high}$/$CD16_{high}$ cells (tube 3) within the lymphocyte population (Fig 4E).

Gating for Isotype Controls

Measurement of background auto-fluorescence and non-specific staining was accomplished using isotype controls (Tubes 1 & 2 – Table 2). Lymphocytes could be gated easily since they do not overlap significantly with other populations. Simple gating of major populations based on SSC and CD45 expression may be adequate (see Fig 1G) for isotype controls; however, gating based on auto-fluorescence (FITC channel) and SSC (Fig 5A) usually provided better discrimination of overlapping granulocyte and macrophage populations. EOS overlap with both PMN and macrophage populations, while DCs are dispersed within the monocyte population. If present in sufficient numbers, the EOS gate could be based on the segregated granulocyte population (Fig 5B). After isolating granulocytes, the remaining monocytes and macrophages could be gated based on differential CD45 and SSC (Fig 5C) Regardless of which approach was used (FITC autofluorescence or CD45 vs. SSC), small populations such as EOS, DCs and basophils (extremely rare) were difficult (or impossible) to isolate without specific antibodies. Figure 5D shows the locations of the EOS and DC populations as determined by specific gating methods using tube 4 (see Fig 3 A and F). A simplistic alternative solution was to “non-specifically” gate the locations where these small populations are expected to exist or to substitute background auto-fluorescence values of their parent populations (i.e. monocytes for DCs, lymphocytes for basophils and PMNs for EOS). This is the approach we have taken for determining background fluorescence for DCs.

Specific Examples for Evaluating Airways Disease and Response to Inhaled Pollutants (Ozone)

The gating strategies and antibody panels described above are useful for monitoring expression of cell surface proteins or quantification and characterization of rare-event populations such as dendritic cells. The usefulness of these strategies for evaluating airways inflammation associated with asthma or exposure to environmental air pollution is demonstrated in Figure 6. As an example, using FCM, a slight yet significant ($p<0.01$)

increase in the proportion of sputum dendritic cells was demonstrated (Fig 6A) in allergic individuals sensitive to dust mites (N=29; 19 mild allergic asthmatics and 10 allergic non-asthmatics) compared to healthy individuals (N=44). The value of flow cytometry in evaluating the effects of air pollutant exposure is exemplified by changes in surface expression of CD14 and HLA-DR on sputum monocytes (Fig 6B) from healthy volunteers 4 hours following a 2-hour inhalation exposure to 0.4 ppm ozone. (Hernandez et al. 2010).

Flow Cytometry-based Differential Cell Counts of Sputum Samples

Proportions of the various sputum leukocyte populations, as estimated by FCM, were similar but not identical to those determined by light microscopy. Differences between the two methods are variable and are affected by multiple factors, including the level of contamination of the sputum sample, cell viability, squamous cell count, and quality of the stained slide. For all major populations, as demonstrated for PMNs in Figure 6, there is generally greater correlation between methods when the sample is minimally contaminated and slide quality is high, as assessed by quality of staining and density of cells on the slide. Based on cell counts derived from tube 4, we found a significant correlation ($p < 0.0001$, Spearman's $r = 0.8233$) between % PMN determined by FCM vs. microscopy (N=45 paired observations). The linear regression line ($R^2 = 0.8856$) and 95% confidence interval are shown (Fig 6C) for a subset of samples (N=30, filled circles) having cell viability ≥ 70% and squamous epithelial cell count ≤ 30% (based on differential counts from stained slides). Remaining samples (N=15, open circles) with higher squamous cell counts and lower viability are also plotted. When all 45 samples are included in the regression, R^2 drops to 0.6658. Similar correlations were found for macrophages (not shown). Lymphocyte proportions, as determined by FCM, tended to be higher than those determined by microscopy (Fig 6D), as were those of EOS (not shown), both of which are present in relatively low proportions compared to PMNs and macrophages.

DISCUSSION

Inherent difficulties and artifacts peculiar to sputum render FCM challenging and necessitate specific gating strategies for both major and rare leukocyte populations such as DCs. Specifically, these include the presence of mucus, squamous and bronchial epithelial cells, dead and degenerate cells, cell detritus, microparticles and exogenous contaminants such as bacteria, yeast, pollen and inhaled dust particles. In addition, the presence of macrophages with inherently high auto-fluorescence necessitates a level of compromise when optimizing instrument settings. Unlike blood, overlapping major sputum leukocyte populations are not readily discernable and are further obscured by contaminating epithelial cells and debris which prevents efficient resolution of leukocyte populations by light scatter properties alone. Here we have outlined methods for overcoming these difficulties, including specific gating strategies for differentiating leukocytes from debris and identification of leukocyte subpopulations using a combination of light scatter properties and differential expression of CD45 and lineage-specific cell surface proteins. In addition, we have provided an approach for evaluating expression of cell surface proteins that are associated with both innate and adaptive immunity.

Samples with low viability are poorly suited for FCM due to excessive contamination with cellular debris, increased non-specific staining and increased auto-fluorescence. Our experience suggests that seventy percent viability or higher (50% at minimum) is desirable for FCM of sputum. Damaged cells may also generate staining artifacts in that labeled antibody may bind, specifically or non-specifically, to fragmented cell membranes and cell debris, or diffuse through leaky cell membranes of intact dead cells. This may allow inclusion of material with low FSC within the CD45(+) gate, thereby producing staining artifact (see Fig1C – F) and erroneous gating. Thus, a combination of both FSC and positive

CD45 expression are required since, individually, neither adequately discriminate leukocytes from contaminants. While the use of isotype control antibody (and perhaps also unlabeled blocking antibody) may help to control for this artifact, the potential for erroneous results remains high when cell viability is low. It might still be possible to gain reliable data from samples with poor viability by segregating them using a viability marker such as propidium iodide (Fig 1E), 7-aminoactinomycin D, Calcein or other dyes. (Barry et al. 2002) Some newer products such as the "fixable" viability stains offered by Invitrogen (LIVE/DEAD® Fixable Dead Cell Stain Kit) may allow routine staining for both viability and surface markers followed by fixation and subsequent acquisition on the flow cytometer the next day. Though we have not yet tried the fixable dyes, they may work well for analysis of sputum samples.

Overlapping expression of various "lineage-specific" proteins on different cell populations may lead to a level of subjectivity in gating populations according to an individual's own style or bias. Although this is unavoidable, subjectivity and bias can be minimized and objectivity increased by including antibodies to more than one differentially expressed marker instead of relying on a single marker. For example, in tube 3, the combination of CD64 and CD16 provides better separation of granulocytes than does CD16, CD14 or CD64 alone. Sequential "Boolean" gating may also be helpful in this respect. Over time, we continue to discover improvements which could be made in our antibody panels and have offered several suggestions as to how inclusion of certain alternate or additional antibodies in a specific tube might provide additional benefit (e.g. the addition of HLA-DR in tube 8). We have presented one approach to gating using tube-specific antibody panels; however, individual investigators can tailor panel configurations to suit their own research needs.

We emphasize that induction/collection and subsequent processing may significantly impact the quality of the sputum sample and subsequent analysis by FCM. Collection and processing of samples should be performed by well-trained and experienced personnel to maximize cell yield and viability and to minimize squamous cell contamination. Careful coaching of the subject/patient during expectoration can significantly reduce squamous cell contamination. While sample filtration will remove some proportion of the squamous cells, it cannot replace good induction technique. We have found that selectively extracting cell-rich mucus "plugs," as opposed to processing the entire sputum sample, recovers the vast majority of the sputum leukocytes, reduces squamous cell contamination and saliva (Pizzichini et al. 1996) and greatly reduces the total volume of the sample to be processed, thereby reducing the dilution effect on fluid phase components of sputum.

Timely sample acquisition following antibody staining (ideally the same day) is desirable to maximize sample quality and minimize gating difficulties due primarily to paraformaldehyde-induced signal decay and increases in auto-fluorescence, both of which may occur over a relatively short period of time (24 – 48 hours). (Stewart et al. 2007) Although logistical issues may prevent same-day sample acquisition, we have found that acquisition within 24 hours of antibody staining does not compromise the integrity of the sample and subsequent data analysis.

Summary

The methods, antibody panels and gating strategies described here were developed over the course of ten years, evolving from two-color FCM, with gating based primarily on light scatter properties, to present day use of multi-color FCM and specific gating strategies which enable more accurate differentiation of sputum leukocytes from debris. Our experience has shown that care must be taken during sputum induction and processing to maximize the quality of the sample for FCM. Specific gating strategies based on a combination of light scatter properties, differential expression of CD45 and lineage-specific

markers are essential for differentiating sputum leukocytes and their subpopulations from squamous epithelial cells and contaminating debris. Due to the significant overlap of major sputum leukocyte populations, it is necessary to include antibody against at least one cell surface marker (preferably two) which, either through positive or negative selection, will facilitate differentiation of granulocytes from mononuclear phagocytes. Once identified, additional FCM-based measurements of leukocyte surface receptors (immunophenotype) and function (phagocytosis, oxidative burst) can be performed. Formulation of antibody panels and gating strategies based on these principles greatly enhance the usefulness of multicolor FCM for the evaluation of sputum leukocytes.

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Abbreviations

FCM	flow cytometry
FSC	forward scatter
SSC	side Scatter
MFI	mean fluorescence intensity
DC	dendritic cell
EOS	eosinophil
PMN	neutrophil
APC	antigen presenting cell
TLR	toll-like receptor
LPS	lipopolysaccharide
BAL	bronchoalveolar lavage
MHC-II	major histocompatibility antigen class II

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Appendix: Extended Methods and Comments

Sputum Sample Collection, Processing and Factors Affecting Sample Quality

Sputum induction and processing techniques vary and are described in detail elsewhere. (Kips et al. 1998b; Kips et al. 1998a; Ronchi et al. 2002; Efthimiadis et al. 2002b; Hargreave et al. 1998; Pizzichini et al. 1996; Spanevello et al. 1998) Methods used here are described in detail elsewhere (Alexis et al. 2000) and are based on the methods of Hargreave et al. (Hargreave et al. 1998) The quality of the sputum sample has major impact on the quality of the flow cytometric assessment and can be influenced both by the induction process and subsequent processing of the sample. Sputum samples should immediately be placed on ice and processed within 2 hours.(Efthimiadis et al. 2002a) Careful processing of the sample is crucial and it is important that both sputum induction and processing be performed by well-trained and experienced personnel to maximize cell yield and minimize squamous epithelial cell and salivary contamination. Excessive or prolonged exposure to dithiothrietol (DTT) during processing adversely affects both cell viability and surface marker expression (Efthimiadis et al. 1997; Loppow et al. 2000) and should be avoided. It is important to note that our laboratory employs the “plug selection” method where cell-enriched mucus “plugs” are manually selected from the surrounding clear saliva fluid and the total selected material is then processed, rather than the “whole sample” method, where the entire raw expectorated sample is processed. The main advantage of the plug-selection method is that it minimizes both squamous cell contamination and dilution from saliva. (Pizzichini et al. 1996; Spanevello et al. 1998; Alexis et al. 2006)

Construction of antibody panels

When constructing antibody panels for sputum analysis, it is essential to include anti-CD45 (leukocyte common antigen, pan leukocyte marker) in all sample tubes to help differentiate leukocytes from debris and facilitate identification of leukocyte populations. We take advantage of major lineage markers such as CD16, CD14, HLA-DR, CD3, CD19, CD56, as well as differential expression of other population specific markers as an aid in isolating specific populations and subpopulations. Certain specific antibodies are included in multiple sample tubes to aid in identifying specific populations for gating purposes. Using these principles, we have developed “standard” panels of fluorescent antibodies (See manuscript Table 2) for identifying leukocyte populations and assessing expression of select cell surface proteins associated with innate and adaptive immunity, antigen presentation and inflammation. Many of the innate immune proteins also interact with the adaptive immune system.

Cytometers equipped with multiple lasers are capable of simultaneously using many more than the five fluorochromes incorporated in our standard 5-color panel. It would be desirable to incorporate more fluorochromes in order to reduce duplication of certain markers, consolidate tubes and conserve cells. We have done this for certain of our more specific protocols, however it is often difficult (short of labeling antibodies in-house) to procure the desired antibody labeled with the necessary fluorochrome to accomplish this. Individual investigators can tailor antibody panels to incorporate additional fluorochromes and to fit their specific research objectives.

Staining Procedures

Cells should be labeled (stained) in a timely manner (as soon as possible) following sample processing and kept at 4 °C in the dark until acquired on the flow cytometer. Data

acquisition should be done within 24 hours for best results.(Stewart et al. 2007) The specific antibody staining procedures have been described elsewhere.(Alexis et al. 2000) We routinely label 100 μl of cell suspension (1×10^6 cells/ml) based on total non-squamous nucleated cells (i.e. leukocytes and bronchial epithelial cells). Following staining, cells were fixed in 1% paraformaldehyde in Dulbecco's phosphate-buffered saline (DPPS). Sources for specific antibodies used in these panels are listed in Table 4..

Specific antibodies were titrated to determine an appropriate amount for staining. The majority of the antibodies were still on the plateau of the titration curve when used at half the manufactures suggested amount (i.e. 10 μl vs. 20 μl). A few (e.g., CD16, CD3) required further dilution. Individual investigators should titrate their antibodies to determine appropriate concentrations for their specific applications. Isotype antibodies also were titrated; however, when we attempted to match protein concentrations and fluorochrome/protein concentrations, we found that the isotype controls had a much higher MFI signal than did certain of the specific antibodies, particularly for the IgG2 isotypes. We therefore found it necessary to dilute isotype control antibodies up to 1:5 (or more). The cause of this disparity is not entirely clear. (Hulspas et al. 2009) Some labs have advocated doing away with isotype controls for this and other reasons (Keeney et al. 1998) while others do not. (O'Gorman and Thomas1999). It may also be useful to employ nonspecific unlabeled blocking antibody to help minimize non-specific staining.

Alternatively, a "cleaner" method for determination of background fluorescence might be the use of "fluorescence minus one" (FMO) analysis (Tung et al. 2004), however, to be done properly, this requires large numbers of cells when multiple populations, multiple specific antibodies, multiple fluorochromes and two different isotypes (IgG1 and IgG2) are used. Since the number of leukocytes obtained from sputum samples is often limited, the FMO approach is usually not feasible for the typical sputum sample. Substitution of blood leukocytes for estimating background fluorescence (Dua et al. 2010) may be adequate for sputum lymphocytes, granulocytes and monocytes; however the absence of macrophages in blood is an obvious problem; although this might be overcome by "spiking" sputum isotype control tubes with blood leukocytes. In our experience, however, sputum monocytes tend to be more granular (higher SSC) with moderately higher autofluorescence (especially in the FITC channel) than peripheral blood monocytes.

Determination of Background Fluorescence Using Isotype Controls

Gating populations in the isotype control tubes is more complex for sputum than for blood since, unlike blood, major non-lymphocyte populations overlap and small or rare populations are imbedded within larger populations. More precise determination of background fluorescence for these small populations, requires inclusion of specific antibodies [e.g. CD9 or CD16 (EOS), CD203c (basophils), or CD14 (DCs)] in addition to CD45 in the isotype control tube.

Mean fluorescence intensity (MFI) of isotype controls (Tubes 1 and 2, Table 2) for specific populations was subtracted from the MFI measured for specific markers to control for background auto-fluorescence and non-specific fluorescence. For individual populations, background fluorescence was determined simultaneously for all fluorescence channels by creating a single histogram and gating 99% of the population (95% at the minimum) in only one fluorochrome channel, usually FITC. As illustrated for the monocyte population (Table 3), background MFIs for the various fluorochromes were relatively constant regardless of which channel was used for gating. This is also true for the other leukocyte populations. This approach avoided the creation of a very large number of histogram gates.

Instrumentation

The majority of our samples are acquired on a BD™ LSR-II digital flow cytometer equipped with 405nm solid state, 488nm argon-ion, and 633nm Helium-Neon lasers, appropriate filters and capability for cross-laser compensation (BD Biosciences Immunocytometry Systems, San Jose, CA). Compensation for spillover and spectral overlap are set for a particular set of instrument settings using BD™ CompBeads (BD Biosciences, cat 552843) and an automated compensation algorithm in BD™ FACSDiva 6.1 software (BD Biosciences). Once established, compensation values are not changed unless the established parameter settings are altered. Our “standard” panel uses 5 colors exciting off the 488 and 633 lasers; however, additional fluorochromes exciting off the 405 laser are used in some studies. This is determined by the availability of specific antibodies in a particular fluorochrome and the amount of sample available. While it is true that less sample is required when more fluorochromes are employed in a particular panel, the trade-off is that the complexity of instrument setup, compensation and data analysis also increases.

Instrument Setup, Data Acquisition and Special Considerations for Sputum Samples

The goal in establishing appropriate instrument settings is to establish the best signal to noise ratio which will provide a measurable signal, while minimizing the coefficient of variation of that signal. Optimal PMT voltages for samples containing mixed populations (e.g. blood) are usually established based on the population having the lowest auto-fluorescence (i.e. lymphocytes) and can be accomplished using cells or beads with low-range fluorescent intensity.(BD Application Note2000) The high auto-fluorescence of sputum macrophages, relative to lymphocytes, requires a compromise to allow application of a single set of PMT voltages for all populations. Our approach has been to optimize instrument settings for monocytes. The monocyte population, labeled only with CD45, is displayed in a separate histogram for each fluorochrome and voltages adjusted to place the entire monocyte population between 200 and 2000, which will place macrophages much higher on the log scale (between 5,000 and 10,000) and lymphocytes somewhat lower, perhaps even straddling zero. In the case of BD™ FACSDiva 6.1 software, bi-exponential plots allow the display of events with fluorescent intensity below zero. It is best, however, to use a setup optimized for lymphocytes if they are of primary interest.

Clinical Relevance

This research describes detailed methods to overcome difficulties associated with flow cytometry of sputum samples, which previously has been lacking in the literature. Flow cytometry of sputum samples can provide valuable information on inflammation and immunological response elements in the bronchial airways for both clinical diagnostic and research applications and can be a useful tool in inhalation toxicology for assessing health effects of inhaled environmental pollutants.

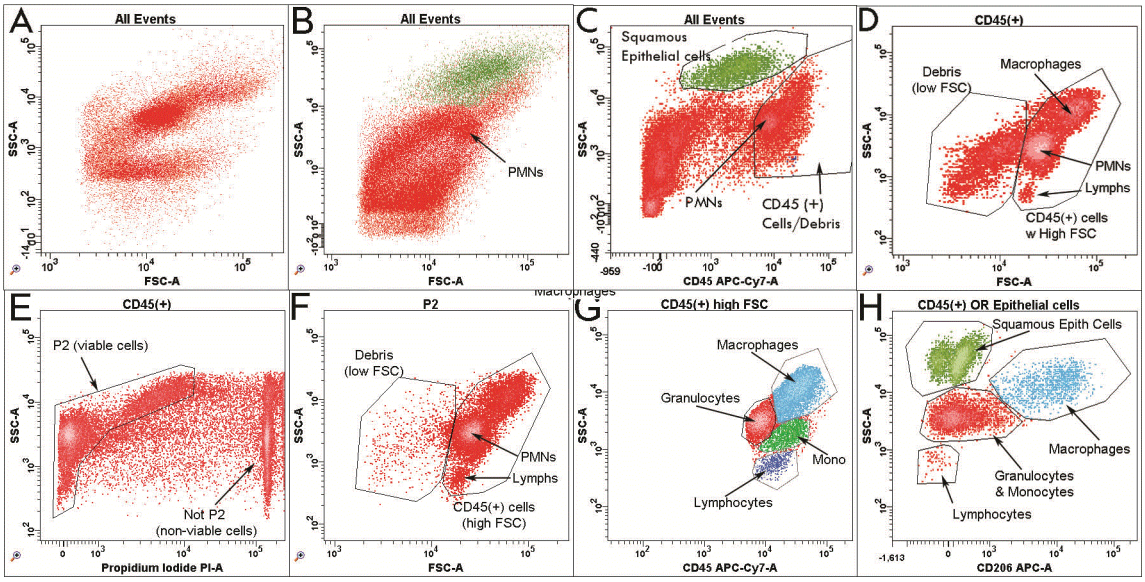

Figure 1. General Gating Strategies for Sputum Leukocyte Identification

(A) Relatively uncontaminated sputum sample. (B) A typical “dirty” raw sputum sample from a mild asthmatic with extensive contamination. Density plots (C – D) more clearly demonstrate location of neutrophils. (C) CD45(+) material including low FSC debris (D) due to non-specific staining or auto-fluorescence. (E) Viable cells were selected (P2) based on negative PI staining. (F) The majority of low FSC material was PI(+) non-viable cells and debris. (G) Cleaned CD45(+) cells showing general locations of cell populations. (H) Squamous epithelial cells are differentiated from macrophages as the CD206 negative population.

SSC = Side Scatter, FSC = Forward Scatter, Mono = monocyte,, PMN = neutrophil, Lymph = lymphocyte

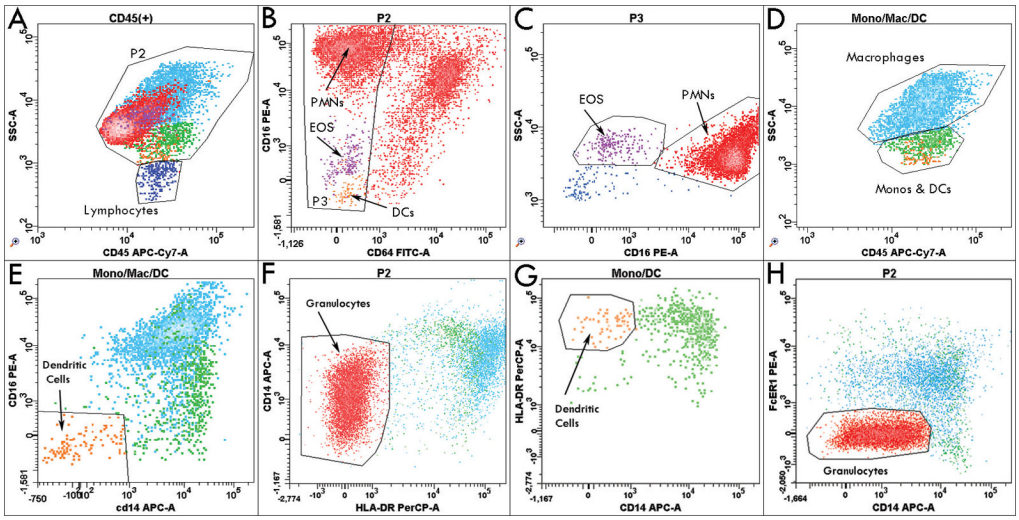

Figure 2. Specific Gating Strategies: (Tube 3, 7, 8) Assessment of Innate Immune Markers

(A) Sputum lymphocytes are gated separately from non-lymphoctes (P2). (B) Differential expression of CD64 and CD16 facilitates separation of granulocytes (P3) from non-granulocytes. (C) Eosinophils are differentiated from PMNs by differential expression of CD16 (tube3). Remaining non-granulocytes from P3 are recombined with “Not P3” to comprise the Mono/Mac/DC population. (D) Macrophages and Mono/DCs gated base on CD45 vs. SSC properties. (E) CD14/CD16 dim dendritic cells (tube 3) fall primarily within the Monocyte gate (D). (F and G) Granulocytes are HLA dim/neg cells in tube 7, while DCs are CD14 dim/HLA high cells within the monocyte gate. (H) Granulocytes are CD14 dim/ FcεR1 dim cells in tube 8.

SSC = Side Scatter, Mono = monocyte, Mac = macrophage. DC = dendritic cell, EOS = eosinophil, PMN = neutrophil,

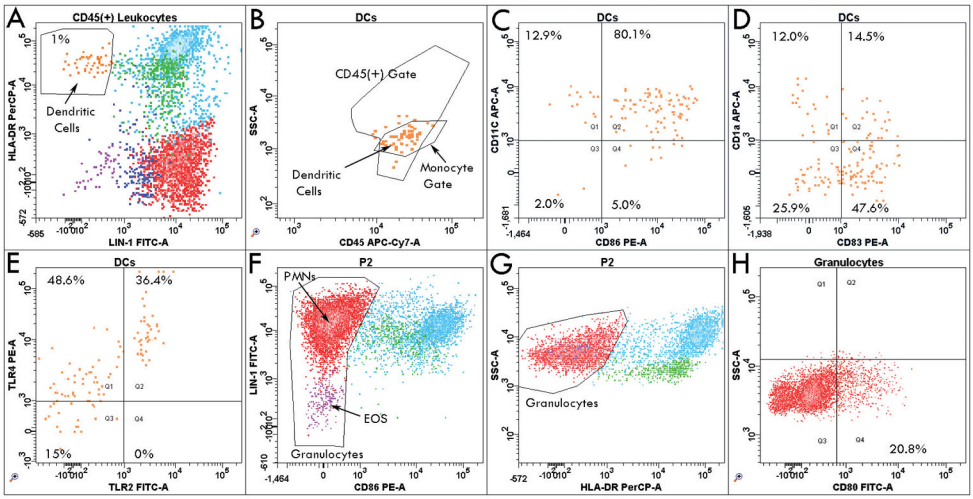

Figure 3. Specific Gating Strategies: (Tube 4, 5 & 6) Assessment of Adaptive Immune Markers

(A) Dendritic cells are lineage-dim/HLA-DR high cells from CD45(+) population and are located primarily within the monocyte gate (B). DCs are primarily CD11c(+) myeloid DCs (C) which express high levels of CD86. (D) The majority of DCs expressed CD83 (tube 5), while relatively few expressed CD1a. (E) Subpopulations of DCs (tube 7) expressed TLR2 and TLR4. Granulocytes gated based on differential expression of CD86 and lineage cocktail (F) or HLA-DR vs. SSC (G). A small proportion of granulocytes may express low levels of CD80 (tube 6) (H).

SSC = Side Scatter, DC = dendritic cell, EOS = eosinophil, PMN = neutrophil

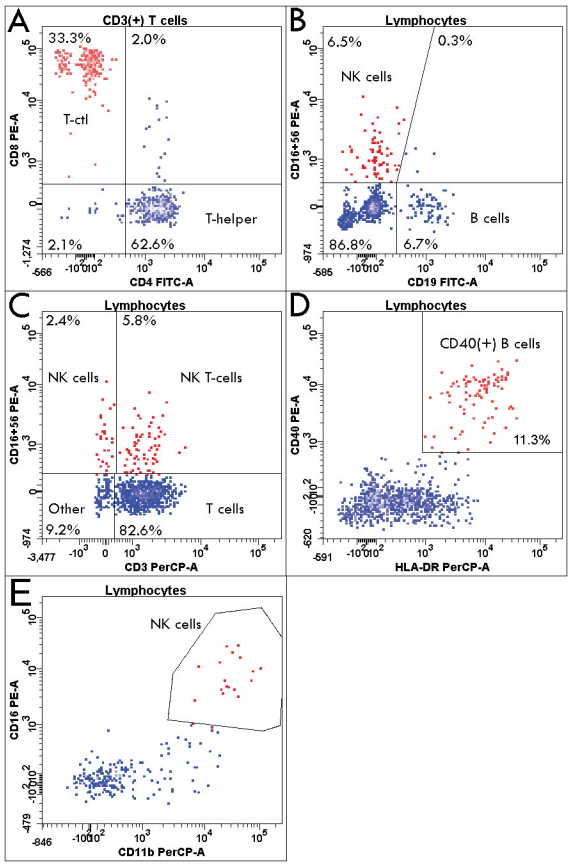

Figure 4. Sputum Lymphocyte Subpopulations

(A) CD3(+) T cells are comprised predominantly of CD4(+) T-helper cells. (B) NK cells and B cells comprise small and variable proportions of the lymphocyte population. (C) CD3(+) NK-T cells comprise the majority of sputum NK cells. (D) Sputum B cells express high levels of CD40. (E) Identification of NK cells (tube 3)

SSC = Side Scatter, FSC = Forward Scatter, NK = natural killer, T-ctl = cytotoxic T cell

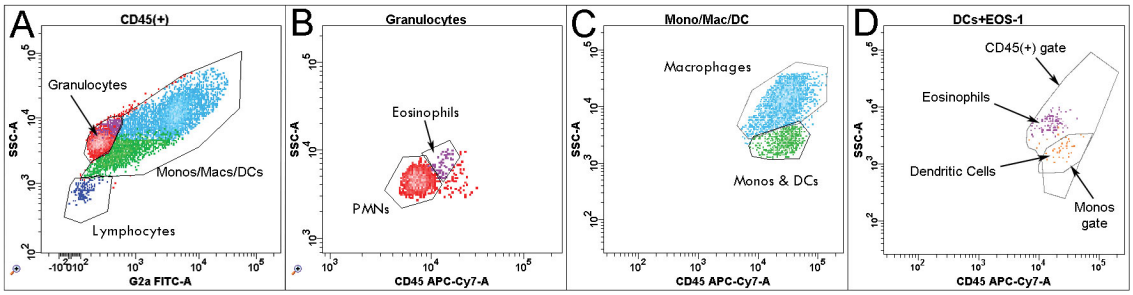

Figure 5. Gating Strategies for Isotype Control Tubes

(A) Gating based on auto-fluorescence (FITC) vs. SSC better separates granulocytes from macrophages than does CD45 (see 1G). (B) EOS gated in the granulocyte population based on CD45 vs. SSC. (C) Gating of monocytes and macrophages (non-granulocytes). (D) Locations of EOS and DCs based on tube 4. Background fluorescence of monocytes is used as a surrogate for DCs.

SSC = Side Scatter, FSC = Forward Scatter, Mono = monocyte, Mac = macrophage. DC = dendritic cell, EOS = eosinophil, PMN = neutrophil

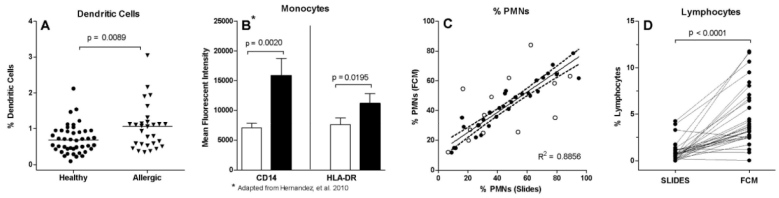

Figure 6. Examples of Data Derived via Flow Cytometry of Sputum

(A) Proportions of sputum dendritic cells was significantly higher in dust-mite sensitive allergic individuals (N=29) compared to healthy non-allergic volunteers (N=44). (B) CD14 and HLA-DR expression was significantly higher on sputum monocytes from healthy volunteers 4hours after a 2-hr inhalation exposure to 0.4 ppm ozone (adapted from (Hernandez et al. 2010). (C) There was a significant (p=0.0001) correlation between %PMNs derived by FCM vs. stained slides (N=45). Filled circles = high viability (>70%) and low squamous cells (<35%). Open circles = low viability or high squamous cells, (D) Lymphocyte proportions tended to be higher based on FCM vs. stained slides.

Table 1

Comparison of Leukocyte Populations of Blood, Sputum and BALF in Healthy Individuals.*

Cell type	Blood	Sputum	BALF
Monos/Macs	2 – 10% (monos)†	30 – 95%	75 – 95%
Dendritic cells	0.5 – 1.0%	0.5 – 1.5%	0.5 – 1.0%
Neutrophils	40 – 75%	5 – 95 %	0 – 1%
Eosinophils	1 – 6%	0 – 1%	0 – 2%
Lymphocytes	20 – 40%	0 – 3%	0 – 23%
Bronchial Epith.	NA	0 – 10%	0 – 3%
Squamous Epith.	NA	1 – 60%	Rare
Basophils	0 – 1%	Extremely Rare	Extremely Rare
Mast cells	0%	0%	0%

*Based on references (Balbi et al. 2007; Bienkowska-Haba et al. 2002; Pizzichini et al. 1998; Grootendorst et al. 1997; Alexis et al. 2000)

†Macrophages are absent from blood. The values for sputum and BALF include monocytes and macrophages combined.

Table 2

Antibody combinations for leukocyte surface proteins associated with adaptive and innate immune responses.

Tube	FITC	PE	PerCP, PE-Cy5	APC	APC - Cy7	Description
1	IgG2a	IgG2a	IgG2a	IgG2a	CD45	Isotype control
2	IgG1	IgG1	IgG1	IgG1	CD45	Isotype control
3	CD64	CD16	CD11b	CD14	CD45	IgG, CR3 and LPS receptor, Innate Immunity
4	Lin-1*	CD86	HLA-DR	CD11c	CD45	Antigen Presentation, Adaptive immunity
5	Lin-1*	CD83	HLA-DR	CD1a	CD45	DC maturity/activation, Adaptive immunity
6	CD80	CD40	HLA-DR	CD206	CD45	Antigen Presentation, Antigen Capture, Innate & Adaptive immunity
7	CD282 (TLR2)	CD284 (TLR4)	HLA-DR	CD14	CD45	Toll-like receptors, Innate immunity
8	CD23 (FcεRII)	FcεRIα	CD19	CD14	CD45	IgE receptor, Adaptive & innate immunity
9	CD4	CD8	CD3	CD25	CD45	Lymphocyte Populations
10	CD19	CD16/56	CD3	CD69	CD45	Lymphocyte Populations

* Lineage Cocktail-1 (BD Biosciences) – a mixture of anti-CD14, 16, 19, 20, 3, and 56.

CD45 is included in all tubes for leukocyte identification and gating. HLA-DR and CD14 are included in multiple tubes to facilitate differentiation and gating of specific populations.

Table 3

Determination of Background Fluorescence Using Isotype Controls.

			Background MFI*			
Population	**Gate Channel**	**% Gated**	**FITC**	**PE**	**PE-Cy5**	**APC**
Monocytes	**FITC**	99.0	880	295	447	717
	PE	99.0	879	283	437	718
	PE-Cy5	99.0	884	286	428	714
	APC	99.9	874	287	440	709
Neutrophils	**FITC**	99.0	311	170	425	734
Macrophages	**FITC**	99.1	7267	3704	4529	776
Lymphocytes	**FITC**	97.8	602	40	286	532

*mean fluorescent intensity

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Table 4

List of antibodies and cellular expression of target molecules.*

Antibody	Clone	Fluorochrome	Source†	Cellular Expression†	Function
CD1a	BL6	APC	Coulter	Mono, Mac, DC, act. T & B cells	Presentation Of Non-Peptide Ag, Cell Mediated Immunity
CD3	13B8.2	PerCP	BD	Pan T cell Marker	Component of T Cell Rcpt. Complex, T Cell Activation
CD4		FICT	Coulter	T-helper cells	Co-Receptor For MHC-II Restricted T Cell Activation
CD8	B9.11	PE	Coulter	Cytotoxic T-cells	Co-Receptor For MHC-I Restricted T Cell Activation
CD11b/CR3	ICRF44	PE-CY5	BD	Gran, Mac, Mono	Phagocytosis, Cell Adhesion
CD11c/CR4	B-ly6	APC	BD	Mac, Mono, Gran	Phagocytosis, Cell Adhesion
CD14	RMO52	APC	Coulter	Mono, Mac, Gran	Lipopolysaccharide (LPS) Receptor
CD16/FcγRIII	3G8	PE	Coulter	PMN, Mac, NK	Phagocytosis, Endocytosis
CD19	J3-119	FITC	Coulter	B cells	B Cell Marker, Component of B Cell Receptor Complex
CD23/FceRII	9P25	FITC	Coulter	Mono, Mac, B cells	Low Affinity IgE Receptor, Negative Feedback on IgE Production
CD25	B1.49.9	APC	Coulter	CD4(+) T-reg, Activated T&B cells	Component of IL-2 Receptor
CD40	MAB89	PE	Coulter	B cells, Mono, Mac, DC,	B Cell Co-Stimulatory Molecule, Antigen Presentation, B Cell Maturation & Survival
CD45	2D1 N901	APC-Cy7	BD	Pan Leukocyte Marker	Leukocyte Common Antigen, B & T Cell Activation & Survival
CD56	(NHK-1)	FITC	Coulter	NK cells, NKT cells	NK Cell Marker, Cell Adhesion
CD64/FcγRI	22	FITC	Coulter	Mac, Mono,	Phagocytosis, Antigen Capture, Antibody Dependent Cellular Cytotoxicity
CD69	FN50	APC	BD	Activated T&B cells, PMNs & EOS	T Cell Activation, Activation Marker
CD80/B7.1	MAB104	FITC	Coulter	Mac, Mono, DC,	Co-Stimulatory Molecule For Antigen Presentation, T Cell Activation
CD83	HB15a	PE	Coulter	Mac, Mono, DC	Co-Stimulatory Molecule For Antigen Presentation?
CD86/B7.2	HA5.2B7	PE	Coulter	Mac, Mono, DC, B cells	Co-Stimulatory Molecule For Antigen Presentation, T Cell Activation
CD206/Mannose Rcpt	19.2	APC	BD	Macrophages	C-Type Lectin Receptor, Phagocytosis, Antigen Capture
CD282/TLR2	TL2.1	FITC	e-Biosc	Mono, Mac, Gran	Pathogen Associated Molecular Pattern (PAMP) Receptor
CD284/TLR4	HTA125	PE	e-Biosc	Mono, Mac, Gran	PAMP (LPS) Receptor
FceRIα	AER-37 (CRA1)	PE	e-Biosc	Mono, Mac, basophils, mast cells,	High-Affinity IgE Receptor, Type I Hypersensitivity
HLA-DR	L243 (G46-6)	PerCP	BD	Mono, Mac, DC, B cells	MHC-II, Antigen Presentation
Lineage Cocktail	Multiple‡	FITC	BD	Non-DC leukocytes	Leukocyte Lineage Marker Cocktail

*There may be significant differential expression of these markers and some leukocytes not listed may express markers only at very low levels or following cell activation.

† Coulter = Beckman Coulter, Inc., Brea, CA; BD = BD Biosciences, San Jose, CA ; e-Biosc = eBiosciences, San Diego, CA; Mac = macrophage; Mono = monocyte; Gran = granulocyte; DC = dendritic cells.

‡ Clones used in Lineage cocktail: NCAM16.2, MφP9, L27, SJ25C1, 3G8, SK7.