Published in final edited form as:

J Immunol Methods. 2007 December 1; 328(1-2): 21–33. doi:10.1016/j.jim.2007.07.019.

A New Approach for Analyzing Cellular Infiltration during Allergic Airway Inflammation

Peter R. Hoffmann^{1,*}, Alexandra Gurary³, Fukun W. Hoffmann¹, Claude Jourdan-Le Saux⁴, Kelsa Teeters¹, Ann C. Hashimoto¹, Elizabeth K. Tam², and Marla J. Berry¹

¹ Department of Cell and Molecular Biology, John A. Burns School of Medicine, University of Hawai'i, Honolulu HI 96813 USA

² Department of Medicine, John A. Burns School of Medicine, University of Hawai'i, Honolulu HI 96813 USA

³ Pacific Center for Emerging Infectious Diseases Research, University of Hawai'i, Honolulu HI 96813 USA

⁴ Pacific Biosciences Research Center, University of Hawai'i, Honolulu HI 96813 USA

Abstract

A mouse model for allergic airway inflammation involving ovalbumin (OVA) sensitization and challenge has been developed that reproduces hallmark features of human asthma and has provided valuable insight into the mechanisms by which this disease occurs. Cellular infiltrate in lungs of mice used in this model have conventionally been evaluated using histological examination of tissue sections and light microscopic analysis of lung lavage samples. As an alternative or complementary approach for characterizing cellular infiltrate, we developed a multicolor fluorescence-activated cell sorter (FACS) method involving the simultaneous detection of seven different markers on lung cell suspensions: CD4, CD8, B220, CD11b, Gr-1, CD49b, and FcεRI. Only some of these cell types increased in OVA-challenged mice compared to PBS controls, including the CD4⁺, B220⁺, CD11b⁺, and FcεRI⁺ groups. We also examined subpopulations of cells for coexpression of these markers and dissected heterogeneous populations as further evaluation procedures to characterize the cellular infiltrate resulting from OVA challenge. Finally, we combined FACS with real-time PCR to analyze certain cell types in terms of mRNA levels for factors involved in asthma, including GATA-3 and IL-1β. Overall, these FACS-based techniques provide a powerful approach for analyzing cellular profiles in lung tissue from mice used in the mouse model of asthma and may also prove valuable in evaluating cellular infiltrates for other models of inflammation and immune responses.

Keywords

Asthma; allergy; airway inflammation; FACS; cellular infiltration

*Corresponding author: Cell and Molecular Biology Department, University of Hawai'i, 651 Ilalo Street, Honolulu, HI 96813, ph: (808) 692-1510, fax: (808) 692-1970, peterh@pbrc.hawaii.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

1. Introduction

Allergic asthma is an inflammatory process driven by inappropriate Th2 immune responses against otherwise innocuous environmental allergens (Umetsu and DeKruyff, 2006). This results in infiltration of inflammatory leukocytes into the lungs, airway hyperresponsiveness, epithelial damage, and tissue remodeling. The immune cells involved in initiating, sustaining, and regulating allergic airway inflammatory responses include monocytes/macrophages, dendritic cells, neutrophils, basophils, mast cells, eosinophils, T and B lymphocytes, and natural killer T cells (Bousquet et al., 1990). In addition, non-leukocytic cells of the lung tissue also contribute to the outcome of the immune responses and remodeling events occurring during asthma (Busse and Lemanske, 2001).

A mouse model for allergic airway inflammation has been developed that reproduces many of the features human asthma and has provided much insight into the mechanisms by which this disease occurs (Lloyd et al., 2001; Tomkinson et al., 2001; McMillan and Lloyd, 2004). The protocol involves sensitizing standard inbred strains of mice such as Balb/c with ovalbumin (OVA) protein absorbed onto aluminum hydroxide via intraperitoneal injections and subsequent intranasal challenges with soluble OVA. Though several variations of this protocol have been used, the mice sensitized and challenged with OVA are typically analyzed within a day of the last challenge.

A critical step in determining the extent of allergic airway inflammation using this model is the full characterization of both the extent and type of cellular infiltration occurring in the lungs. Cellular infiltration characterization is typically accomplished by analyzing cells washed from the lungs in the BAL. Enumeration of total cells in the BAL may be performed using a manual method involving a hemacytometer and a light microscope or using an automated Coulter particle counter. Once the total number of cells in the BAL has been measured, the profile of the cell types present in the BAL is often determined using cytocentrifugation of BAL onto glass slides followed by differential staining. Cytospin preparations commonly utilize non-specific staining procedures such as Wright-Giemsa (differential) stains that facilitate visualization of the phenotypes of the cells washed from the airways. Cellular features including size, cytoplasmic granularity, and nuclear appearance allow identification of different cell types including eosinophils, lymphocytes and others.

These conventional approaches to analyzing the extent of cellular infiltration and cell types that make up that infiltrate have limitations. First of all, cytopins do not allow for a clear, objective distinction between all cell types. Eosinophils are the most abundant cell type found in the OVA-challenged lung and have a distinctive phenotype. However, neutrophils may sometimes be mistaken for eosinophils due to some structural similarities and particular attention must be paid to minor differences in phenotypic features to avoid errors. Also, while lymphocytes can be clearly distinguished from other cell types using the cytospin technique, T and B cells cannot be distinguished from each other. Furthermore, subtypes of T cells such as CD4⁺ helper T cells versus CD8⁺ cytotoxic lymphocytes (CTLs) cannot be distinguished. Finally, an issue that arises with cytospin techniques is that cells often do not fit the criteria of eosinophils, neutrophils, or lymphocytes and thus are inconsistently categorized. These cells are sometimes described as macrophages, monocytes/macrophages, mast cells, or simply as "others." In fact, they often are not included in the cellular profile of the BAL due to a lack of ability of researchers to accurately phenotype them. This may result in studies that are difficult to compare to each other or difficult to interpret.

In addition to interpretive and reporting inconsistencies, there are technical issues regarding the use of BAL to characterize the cellular infiltrate in the lungs. The washing of the lungs with saline (e.g. PBS) removes those cells that are not embedded within the tissues of the lung and

may not give a true representation of the total cellular infiltrate. Also, in the BAL there is a distinct paucity of lung tissue cells such as epithelial, endothelial, fibroblast, myofibroblast, and smooth muscle cells, all of which need to be included for a full characterization of cellular responses in allergic asthma. These issues are compounded by the technical problem that may arise if the saline is not fully perfused into the small airways and fully withdrawn. For example, washing the upper airways while leaving saline remaining in the lower airways may result in an inaccurately low cell count and aberrant cellular profile determination.

As an alternative or complementary approach to enumerate and phenotypically characterize the cellular infiltrate that results using the mouse model of allergic airway inflammation, we developed a multi-color fluorescence-activated cell sorter (FACS) approach. While FACS-based approaches have been used to a limited extent to evaluate lung inflammation during asthma (Bischof et al, 2003; Gwinn et al, 2006; Webb et al, 2007), major advances have been made in the development of a variety of fluorochromes, monoclonal antibodies against cellular markers, and cytometric instrumentation for collecting data, all of which have greatly expanded the utility of FACS technology. Herein we report our use of a FACS-based approach for analyses of cellular infiltrate resulting from allergic airway inflammation as well as the interesting results obtained using this approach. We found that the FACS-based method provided extensive information on the cell types infiltrating and/or expanding in the lung in response to OVA challenge. It also provided a means for sorting subpopulations for molecular analysis of factors involved in this disease including GATA-3 and IL-1 β . In this manner, we believe that this approach may prove useful for researchers utilizing the mouse model of allergic airway inflammation to more fully characterize the cells involved in this disease.

2. Methods

2.1. Mice

Balb/c mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA) and were maintained at the animal housing facility at the John A. Burns School of Medicine at the University of Hawai'i. All animal experimental protocols were approved by the University of Hawai'i Institutional Animal Care and Use Committee.

2.2. Mouse model of allergic airway inflammation

Male mice of 8–10 weeks of age were sensitized by intraperitoneal (i.p) injections on day 0, 7 and 14 with 50 μ g of ovalbumin (OVA) (Sigma-Aldrich, St. Louis, MO) precipitated with 0.5 mg of aluminum hydroxide in 200 μ l of PBS. The mice were then challenged by administering 50 μ g OVA in 50 μ l PBS directly into the nostrils using a micropipetor on days 21, 23, and 25 using isofluorane as an anesthetic. Negative control mice were sensitized and challenged with PBS alone. On day 26, the mice were sacrificed and various tissues collected for analyses. Pilot experiments were conducted that included control mice sensitized with either alum alone or OVA/alum followed by PBS-challenge. These mice were found to have levels of lung inflammation similar to that found in mice that received PBS for both sensitization and challenges (data not shown). Therefore, control mice from the latter group were used for all subsequent experiments.

2.3. Bronchoalveolar lavage

BAL was collected by injecting and withdrawing 0.8 ml cold PBS. Recovered BAL (70–80%) was centrifuged at 300 g for 8 min. The cells were resuspended in 0.5 ml PBS and adhered to glass slides using cytocentrifugation. Slides were stained using Wright-Giemsa and visualized using a Zeiss Axioscope light microscope.

2.4. Lung histology

Lung tissue was prepared for histological analyses by fixation in 10% buffered formalin overnight, followed by a PBS wash and step-wise dehydration in 60–100% ethanol. The tissue was then incubated in xylene, embedded in paraffin, sectioned, and stained with a standard Hematoxylin and Eosin (H&E) staining method.

2.5. Fluorescence-activated Cell Sorting and Flow Cytometric Analyses

Lungs were removed, washed with PBS to remove blood, tissue minced and incubated for 1 h at 37°C in digestion buffer consisting of RPMI with 10% FBS (GIBCO/Invitrogen, Carlsbad, CA), 0.5 mg/ml Liberase CI and 30 µg/ml DNase I (both from Roche Applied Science, Indianapolis, IN). After digestion, the lung tissue was forced through a 40 µm cell strainer, cells pelleted by centrifugation, and erythrocytes lysed with RBC Lysis Buffer (Sigma). The cells were washed once more with RPMI and resuspended in 500 µl PBS containing 2% FBS on ice. Aliquots of 10 µl were removed for enumeration using a Coulter particle counter. For cell sorting, the entire sample was transferred to FACS tubes topped with cell filters to remove clumps and suspended in a final volume of 500 µl FACS buffer (PBS containing 2% FBS) on ice. For samples that were not intended for cell sorts, but only for fluorescence analysis, 1×10^6 cells were transferred to the same FACS tubes in a total of 200 µl FACS buffer on ice. The cells were then incubated with Fc-block (BD Pharmingen, San Diego, CA) at a final concentration of 1:200 for 20 min on ice. A cocktail of the following antibodies was then added (clone number; final concentration used for cell sorts; final concentration used for fluorescence analyses): FITC-anti- ϵ FcRI (MAR-1; 3.125 µg/ml; 1.25 µg/ml), PE-anti-CD8 (53-6.7; 7.5 µg/ml; 3 µg/ml), and PE/Cy7-anti-CD49b (DX5; 15 µg/ml; 6 µg/ml) (all purchased from Ebioscience, San Diego, CA); APC-anti-CD4 (RM4-5; 15 µg/ml; 6 µg/ml), APC/Cy7-anti-CD11b (M1/70; 7.5 µg/ml; 3 µg/ml), PerCP-Cy5.5-anti-Gr-1 (RB6-8C5; 15 µg/ml; 6 µg/ml), and PE/Texas Red-anti-B220 (RA3-6B2; 15 µg/ml; 6 µg/ml) (all purchased from BD Pharmingen). Isotype control antibodies were tested at the same concentrations and staining intensity was not found to change between PBS- and OVA-challenged mice. After 40 min incubation on ice, cells were washed and analyzed on a FACSAria fluorescence-activated cell sorter (BD Biosciences). For each sample, up to four fluorochrome-tagged cell types were sorted and collected, while the remainder of fluorochrome-tagged cell types were not sorted but were included in analyses. In some cases, no samples were sorted and all fluorochrome-tagged cell types were subjected only to analyses. BD CompBeads Compensation Particles were used to set the compensation during data acquisition and all analyses were carried out using FACSDiva 6.0 software (BD Biosciences).

2.6. RNA extraction and real-time PCR

Sorted cells were collected into RPMI media containing 10% FBS. These cells were then centrifuged at 1,200 rpm for 6 min, media removed, and cell pellets frozen at –80°C until later use. Cell pellets were thawed and RNA extracted using an RNeasy Mini kit and RNase-free DNase I (all from Qiagen, Valencia, CA). The eluted RNA was precipitated by adding 100% ethanol (2.5-times elution volume), 3M sodium acetate (0.1-times elution volume), and glycogen (0.01-times elution volume), incubating overnight at –80°C, and pelleting by centrifugation at 12,000 rpm for 30 min. Supernatant was removed and cell pellets washed with cold 80% ethanol, followed by another centrifugation at 12,000 rpm for 10 min. The ethanol was removed, and the RNA pellets dried, then resuspended in 10–15 µl water. Concentration and purity of RNA was determined using A_{260}/A_{280} measured on an ND1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE). Synthesis of cDNA was carried out using Superscript III (Invitrogen) and oligo dT primer with 80 ng RNA per 20 µl reactions. For real-time PCR, 3.5 µl of the cDNA was used in 10 µl reactions using Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen) carried out in a LightCycler 2.0 thermal

cycler (Roche). Oligonucleotides used for PCR included primers specific for hypoxanthine phosphoribosyltransferase (HPRT) (fwd: 5'-TCCTCCTCAGACCGCTTTT-3'; rev: 5'-CCTGGTTCATCGCTAATC-3') and commercial proprietary oligonucleotides specific for murine GATA-3 (cat# PPM05199A; GenBank reference # NM_008091) and murine IL-1 β (cat# PPM03109A; GenBank reference # NM_008361), purchased from Superarray, Inc (Frederick, MD). Cycling conditions were used as suggested in the SYBR Green kit instructions and results analyzed using Relative Quantification Software (Roche).

2.7. Statistical Analyses

All statistical tests were performed using GraphPad Prism version 4.0 for Windows (GraphPad Software, San Diego, CA). Means of two groups were compared using a student's t test and significance considered at $p < 0.05$.

3. Results

3.1. Multicolor flow cytometric analysis of lung cells in OVA-challenged mice versus PBS controls

Conventional techniques for analyzing cellular infiltrate of airways during allergic inflammation involving either histological analyses of tissue sections or cytocentrifugation preparations of BAL methods typically produce results as those displayed in Figure 1A and 1B, respectively. These methods clearly demonstrate the success of the allergic challenge protocol and produce helpful, albeit limited, information regarding the phenotypic features of the cells washed out of the airways during the lavage. In particular, the cytocentrifugation followed by differential staining allow identification of eosinophils and lymphocytes due to their distinguishing phenotypic features, while other cell types are not as reliably identified. For example, an evaluation of the types of lymphocytes responding to the OVA challenge is not possible with this conventional technique. This technique has produced limited data regarding the relative numbers of B cells to T helper cells to cytotoxic T cells infiltrating the airways. In contrast, the use of multicolor flow cytometric analyses allow the simultaneous identification of several different cell types in the lung (Figure 1C–E). For this study, we identified surface markers for seven different types of leukocytes including T cells (CD4 and CD8), B cells (B220), mast cells and basophils (Fc ϵ RI), granulocytes (Gr-1), natural killer cells (CD49b), and monocytes/macrophages (CD11b). Analysis of the data was carried out in several steps and revealed several interesting features. As an initial step for analyzing the stained lung cells, a gate was constructed in the forward-scatter (FSC) versus side-scatter (SSC) dot plot for identifying the cells with the size and granularity properties of lymphocytes, which appear as red dots in the subsequent dot plots. Cells that stained for CD4, CD8, and B220 exhibited the lymphocyte FSC and SSC properties and appeared red in color within each of the gates drawn for these markers (Figure 1B–D). Only some of the cells that stained for CD49b and CD11b exhibited the lymphocyte FSC and SSC properties, while none of the cells within the Fc ϵ RI or Gr-1 exhibited these properties.

To gain insight into the relative abundance of each cell-type, gates were drawn around the populations staining positive for each marker and the percent of each cell-type present in the lung cell sample was evaluated. When 30,000 lung cells were evaluated for seven different surface markers, a significant increase in percent abundance in OVA versus control was found for several cell types including T helper cells (CD4⁺), B cells (B220⁺), mast cells/basophils (Fc ϵ RI⁺), and monocytes/macrophages (CD11b⁺) (Figure 1E). Cell types that did not increase in relative abundance included CTLs (CD8⁺), granulocytes (Gr-1⁺), and NK cells (CD49b⁺).

These FACS analyses should also include a vital fluorescent dye staining control to determine the viability of the cells that are being evaluated for surface markers. Ideally, dead cells would

be identified using 4'-6-Diamidino-2-phenylindole (DAPI), which is excited by a blue laser line and emits in the blue fluorescence channel. This fluorescence could be used simultaneously with the other fluorochromes and dead cells (DAPI-positive) could then be omitted from the multicolor analyses. Because our cell sorter was not equipped with a blue laser line, we analyzed the viability separately from our cell surface markers using a different vital fluorescent dye, propidium iodide (Figure 1F). Our results revealed that 4.4% of total cells were propidium iodide-positive, thus 4.4% cells included in our analyses were not viable cells. This is an important aspect of FACS-based experiments that must be taken into consideration when performing and interpreting the results.

A limitation in presenting these data is that the relative quantity of each cell type is obtained, not absolute numbers. Obtaining absolute numbers is possible with this method and would require consistent and complete dissection of lung tissue from mouse to mouse, accurate enumeration of cell suspensions obtained from lung digestion reactions, and normalization to total mouse mass. Other newly developed techniques are available for accurate quantitative data to be obtained using flow cytometry such as bead-enhanced cytometric methods (Montes et al., 2006), but the study presented herein deals only with relative quantities of different cell types within the lung tissues.

3.2. Coexpression of B220 and CD11b

One of the groups of cells demonstrating a significant increase in percent abundance in lung when comparing OVA-challenged mice to PBS controls was the B220-positive population. The infiltration of B cells into lung tissue during allergic airway inflammation has not been well-studied using conventional BAL techniques and in general remains a relatively unexplored area of investigation. In fact, studies using this mouse model of asthma have demonstrated that B cells are not essential for development of eosinophilia or airway inflammation (Korsgren et al., 1997). B220 is a relatively specific marker for B cells in mice, but has also been shown to be expressed at lower levels on activated T cells and subsets of dendritic cells. Given the fact that the B220-positive cells predominantly consisted of cells with low FSC and SSC properties, the B220-positive cells increasing in response to OVA challenge were unlikely to be dendritic cells. At least two populations of B220-positive cells were found to increase in the lung in response to OVA-challenge: B220^{lo} and B220^{hi} (Figure 2A). We next utilized the multicolor features of the technique described in this study and found both of these populations increased in coexpression of CD11b upon OVA-challenge compared to PBS controls. For B220^{hi} cells in particular, analysis of the B220^{hi}CD11b⁻ and B220^{hi}CD11b⁺ subsets showed that both increased in response to the OVA challenge (Figure 2B). Both subsets increased in equivalent amounts: 239% B220^{hi}CD11b⁻ and 250% B220^{hi}CD11b⁺ in OVA-challenged mice compared to PBS controls. However, only the B220^{hi}CD11b⁻ population increase was statistically significant.

These data suggest that B220-positive cells are a major constituent of the cellular infiltrate occurring during allergic airway inflammation and these cells may represent B cells and/or activated T cells. Some of these B220-positive cells co-express CD11b, and exactly what their role is in the inflammatory processes that occur during allergic asthma needs further investigation. Overall, this exercise demonstrates that analyzing cell populations that co-express surface markers may provide valuable insight into as-of-yet unidentified cell types that contribute to the cellular infiltrate during inflammatory processes.

3.3. Using multicolor flow cytometry to analyze highly heterogeneous populations

CD11b (also called α M or Mac-1) is an integrin chain that, together with CD18 (β 2 integrin), functions as a receptor for complement (C3bi), intercellular adhesion molecule-1 (ICAM-1), fibrinogen, or clotting factor X (Altieri et al., 1986; Altieri et al., 1988; Wright et al., 1988;

Diamond et al., 1991). CD11b is often used as a monocyte/macrophage marker, but is also expressed on a variety of other cell types including neutrophils and monocytes (Springer et al., 1979) as well as activated CD8⁺ T cells (McFarland et al., 1992) and NK cells (Ault and Springer, 1981). Our results demonstrated that CD11b⁺ cells were significantly increased in lung tissue when comparing OVA-challenged mice to PBS controls. Within the CD11b⁺ group, there was a large degree of heterogeneity when CD11b-staining intensity was plotted versus SSC property (Figure 3A). Thus, we utilized the power of the multicolor flow cytometric technique to further investigate the sub-populations within the CD11b⁺ group in the OVA-challenged mice and PBS controls. As a first step, we displayed each cell-type individually in dot plots to determine which of these cell types appeared in the CD11b⁺ gate (Figure 3B). Results indicated that a subpopulation of lymphocytes stained positive for CD11b and that these were predominantly B220⁺ cells and, to a lesser extent, CD4⁺ cells. Relatively few CD8⁺ lymphocytes were found to stain for CD11b. Appearing within the CD11b⁺ gate were also cells staining positive for the markers Gr-1, FcεRI, and CD49b. The CD11b⁺Gr-1⁺ and the CD11b⁺FcεRI⁺ showed both SSC^{lo} and SSC^{hi} properties, while the CD49b⁺CD11b⁺ cells were predominantly SSC^{lo}. In fact, the cell subpopulation in the lower portion of the CD11b⁺ gate was found to consist of cells co-expressing CD11b, Gr-1, and CD49b (multicolor analyses not shown). To quantify differences between PBS- and OVA-challenged mice within the CD11b⁺SSC^{lo} and CD11b⁺SSC^{hi} subpopulations, gates were constructed and cell numbers determined for each subpopulation (Figure 3C and D). Results showed that both CD11b⁺SSC^{lo} and CD11b⁺SSC^{hi} increased when comparing OVA- to PBS-challenged mice. However, the increase for the CD11b⁺SSC^{hi} group (5.13 times) was greater than that for the CD11b⁺SSC^{lo} group (2.0 times). This exercise demonstrates the powerful approach of dividing heterogeneous populations into subpopulations for comparing treated animals to controls. In particular, our initial analyses showed that Gr-1⁺ and CD49b⁺ cells did not increase with OVA-challenge (Figure 1E), but a detailed evaluation showed that both the CD11b⁺Gr-1⁺ (cells within the CD11b⁺SSC^{hi} gate) and the CD11b⁺CD49b⁺Gr-1⁺ population (cells within the CD11b⁺SSC^{lo} gate) significantly increased with OVA-challenge.

3.4. Molecular analysis of sorted cells

Another powerful feature of the multicolor flow cytometric approach is that cell types of interest may be sorted and collected during FACS analyses of lung tissue. As proof of principle, we carried out an experiment in which lung cells from either PBS- or OVA-challenged mice were stained as described above and cells positive for either CD4 or CD11b were sorted into separate tubes. Levels of mRNA were determined for two important gene products involved in inflammation: 1) GATA-3 in the CD4⁺ cells (Zhu et al., 2006) and 2) IL-1β in the CD11b⁺ cells (Ichiki et al., 2005). Our results clearly demonstrate the ability to determine mRNA abundance for both GATA-3 and IL-1β relative to the housekeeping gene product, hypoxanthinephosphoribosyltransferase (hpert). When comparing OVA-challenged mice to PBS controls, mRNA levels for the Th2-inducing transcription factor, GATA-3, were found to increase in CD4⁺ cells and mRNA levels for the Th1 cytokine, IL-1β, were found to decrease in CD11b⁺ cells. Although quantities of total RNA extracted from sorted cells were relatively low (< 100 ng per mouse), the quantity was sufficient to carry out analyses of several different target mRNAs. Alternatively, total RNA extracted from mice within one experimental group may be pooled to increase the quantity of mRNA that may be analyzed in one experiment.

4. Discussion

The allergic airway inflammatory processes that drive asthma involve many different cell types acting in a complex manner to aberrantly respond to seemingly innocuous antigens. Understanding the mechanisms that initiate, maintain, and drive asthma will require a full characterization of the cells involved. We have developed a new approach to studying the

cellular populations that infiltrate lung tissue during this process. Our data suggest that this method provides reproducible results that allow simultaneous characterization of multiple cell types infiltrating lung tissue during allergic airway inflammation. We evaluated lung tissue cells for relative quantities of seven different markers and identified four cell types that were significantly higher in OVA-challenged mice compared to PBS controls: CD4⁺ lymphocytes, B220⁺ lymphocytes, FcεRI⁺ cells, and CD11b⁺ cells. Overall, our study presents an alternative or complementary approach for analyzing the cellular infiltrate in lungs of mice used in an increasingly popular mouse model for asthma. In fact, the approach described herein may be adapted and utilized for other mouse models of inflammation such as experimental colitis, 1-chloro-2,4-dinitrobenzene (DNCB)-induced allergic contact dermatitis, infectious disease models, or other inflammatory models.

A multitude of studies have focused on determining the genetic or molecular characteristics of lung tissue during allergic asthma (reviewed in (Umetsu and DeKruyff, 2006) and (Bochner and Busse, 2005)). Important discoveries have been made for genes or proteins that modify airway responsiveness in asthmatic patients including ADAM33, ADRB2, and eotaxin (Park et al., 2006). Crucial information regarding the particular cell types that express these gene products during acute and/or chronic asthma may be obtained using the mouse models of allergic airway inflammation. One of the powerful features of the approach presented in this study is that sorting cells of interest and analyzing their extracted nucleic acid or protein may allow a dissection of sources of certain causative or protective factors. In this manner, a more precise picture may emerge regarding the roles various cells play in different steps of the immune responses and inflammatory events occurring during asthma.

Another downstream application that may be combined with the FACS-based approach described herein is the adoptive transfer of cells obtained from sorts. Adoptive transfers have frequently been carried out either during or before the sensitization and challenge steps of the mouse model of asthma (Janssen et al., 2000; Goya et al., 2003; Beier et al., 2004). The rationale for this often is to investigate the effects that certain cells may have on either mitigating or exacerbating inflammatory outcome or, in some cases, to rescue the inflammatory process in certain knockout mice to show the necessity of certain cell types for the disease process. During our study, we obtained sufficient numbers of CD4⁺ T cells, B220⁺ cells, and other cell types sorted from OVA-challenged lungs for use in adoptive transfer studies. Thus, extending the protocol described in our study to include adoptive transfer techniques may allow evaluation of cellular profiles in the lung during asthma and the simultaneous collection of some of these cells for transfer into recipient mice.

Although FACS-based techniques have several advantages over more traditional techniques for analyzing allergic airway inflammation, it must be emphasized that FACS-based techniques have their own set of limitations. The most obvious of limitations is the availability and cost of a FACS instrument for use by the investigators. The utility and power of a dual- or triple-laser equipped FACS instrument may not offset the expense of the instrumentation and personnel necessary for acquiring and establishing this technology. The fluorochrome-conjugated antibodies are also costly compared to simple differential stains. Another limitation of the technique is the number of animals that may be analyzed at one time. Because cell sorts take time, careful planning must be made for this technique to be used efficiently for acquisition of sufficient data for testing hypotheses. We found that up to four mice could be analyzed in a full day, with each set of mouse lungs (up to 18 million total cells) taking approximately two hours to fully sort. A full study would require multiple days of tissue processing and cell sorting, but this holds true for most applications involving cell sorts. A final limitation that was described in the results section above that is worth repeating is the fact that relatively small quantities of nucleic acid or protein are likely to be isolated from sorted lungs. We were typically able to isolate small quantities of total RNA (< 100 ng) from lungs of OVA-challenged

mice, while microgram quantities may be required for extensive downstream analyses. Pooling RNA from animals within experimental or control groups is an approach that may allow one to overcome shortages of quantities, but requires the use more animals.

It should also be noted that the approach described in this study provides limited information regarding eosinophil recruitment, one of the key features in allergic lung inflammation. Eosinophils are one of the cell types known to express Gr-1, but our initial analyses showed that Gr-1⁺ cells did not increase with OVA-challenge (Figure 1E). The eosinophil populations may constitute a substantial portion of the CD11b⁺Gr-1⁺ or CD11b⁺CD49b⁺Gr-1⁺ populations that significantly increased with OVA-challenge (Figure 3B and C). This highlights one advantage of including conventional differential cell counts of BAL, i.e. characterizing eosinophil recruitment. In fact, enumeration of BAL eosinophils may be combined with FACS analyses of lung tissue to provide comprehensive characterization of lung infiltrate.

The study presented in this paper was borne out of our desire to find a new method for analyzing cellular infiltrate in lungs of mice used in the mouse model of acute asthma. We believe that the data presented herein show the FACS-based approach to be powerful. The techniques described in this report may be modified or adapted to fit the goals of the studies in which it is implemented. Overall, these techniques may be considered by investigators using this and other mouse models to study cell types involved in various inflammatory processes and may lead to even more sophisticated approaches in the search for treatment and prevention measures for these diseases.

Acknowledgments

This publication was made possible by Grant Number G12 RR003061 and P20RR016467 from the National Center for Research Resources (NCRR), a component of the National Institutes of Health (NIH). Its contents are solely the responsibility of the authors and do not necessarily represent the official view of NCRR or NIH. This work was also supported by NIH award R01 DK40302, a Hawai'i Community Foundation Grant, and the American Lung Association of Hawai'i.

Abbreviations used

BAL	bronchioalveolar lavage
OVA	ovalbumin
FACS	fluorescence-activated cell sorter
CTLs	cytotoxic lymphocytes
FSC	forward-scatter
SSC	side-scatter
DAPI	4'-6-Diamidino-2-phenylindole

References

- Altieri DC, Mannucci PM, Capitanio AM. Binding of fibrinogen to human monocytes. *J Clin Invest* 1986;78:968–76. [PubMed: 3760194]
- Altieri DC, Morrissey JH, Edgington TS. Adhesive receptor Mac-1 coordinates the activation of factor X on stimulated cells of monocytic and myeloid differentiation: an alternative initiation of the coagulation protease cascade. *Proc Natl Acad Sci U S A* 1988;85:7462–6. [PubMed: 2971972]
- Ault KA, Springer TA. Cross-reaction of a rat-anti-mouse phagocyte-specific monoclonal antibody (anti-Mac-1) with human monocytes and natural killer cells. *J Immunol* 1981;126:359–64. [PubMed: 7451976]

- Beier KC, Hutloff A, Lohning M, Kallinich T, Kroczeck RA, Hamelmann E. Inducible costimulator-positive T cells are required for allergen-induced local B-cell infiltration and antigen-specific IgE production in lung tissue. *J Allergy Clin Immunol* 2004;114:775–82. [PubMed: 15480315]
- Bischof RJ, Snibson K, Shaw R, Meeusen EN. Induction of allergic inflammation in the lungs of sensitized sheep after local challenge with house dust mite. *Clin Exp Allergy* 2003;33:367–75. [PubMed: 12614452]
- Bochner BS, Busse WW. Allergy and asthma. *J Allergy Clin Immunol* 2005;115:953–9. [PubMed: 15867851]
- Bousquet J, Chanez P, Lacoste JY, Barneon G, Ghavanian N, Enander I, Venge P, Ahlstedt S, Simony-Lafontaine J, Godard P, et al. Eosinophilic inflammation in asthma. *N Engl J Med* 1990;323:1033–9. [PubMed: 2215562]
- Busse WW, Lemanske RF Jr. Asthma. *N Engl J Med* 2001;344:350–62. [PubMed: 11172168]
- Diamond MS, Staunton DE, Marlin SD, Springer TA. Binding of the integrin Mac-1 (CD11b/CD18) to the third immunoglobulin-like domain of ICAM-1 (CD54) and its regulation by glycosylation. *Cell* 1991;65:961–71. [PubMed: 1675157]
- Goya I, Villares R, Zaballos A, Gutierrez J, Kremer L, Gonzalo JA, Varona R, Carramolino L, Serrano A, Pallares P, Criado LM, Kolbeck R, Torres M, Coyle AJ, Gutierrez-Ramos JC, Martinez AC, Marquez G. Absence of CCR8 does not impair the response to ovalbumin-induced allergic airway disease. *J Immunol* 2003;170:2138–46. [PubMed: 12574386]
- Gwinn WM, Damsker JM, Falahati R, Okwumabua I, Kelly-Welch A, Keegan AD, Vanpouille C, Lee JJ, Dent LA, Leitenberg D, et al. Novel Approach to inhibit asthma-mediated lung inflammation using anti-CD147 intervention. 2006;177:4870–79.
- Ichiki H, Hoshino T, Kinoshita T, Imaoka H, Kato S, Inoue H, Nakamura H, Yodoi J, Young HA, Aizawa H. Thioredoxin suppresses airway hyperresponsiveness and airway inflammation in asthma. *Biochem Biophys Res Commun* 2005;334:1141–8. [PubMed: 16039995]
- Janssen EM, van Oosterhout AJ, Nijkamp FP, van Eden W, Wauben MH. The efficacy of immunotherapy in an experimental murine model of allergic asthma is related to the strength and site of T cell activation during immunotherapy. *J Immunol* 2000;165:7207–14. [PubMed: 11120853]
- Korsgren M, Erjefalt JS, Korsgren O, Sundler F, Persson CG. Allergic eosinophil-rich inflammation develops in lungs and airways of B cell-deficient mice. *J Exp Med* 1997;185:885–92. [PubMed: 9120394]
- Lloyd CM, Gonzalo JA, Coyle AJ, Gutierrez-Ramos JC. Mouse models of allergic airway disease. *Adv Immunol* 2001;77:263–95. [PubMed: 11293118]
- McFarland HI, Nahill SR, Maciaszek JW, Welsh RM. CD11b (Mac-1): a marker for CD8+ cytotoxic T cell activation and memory in virus infection. *J Immunol* 1992;149:1326–33. [PubMed: 1500720]
- McMillan SJ, Lloyd CM. Prolonged allergen challenge in mice leads to persistent airway remodelling. *Clin Exp Allergy* 2004;34:497–507. [PubMed: 15005746]
- Montes M, Jaensson EA, Orozco AF, Lewis DE, Corry DB. A general method for bead-enhanced quantitation by flow cytometry. *J Immunol Methods* 2006;317:45–55. [PubMed: 17067632]
- Park HS, Kim SH, Park CS. The role of novel genes in modifying airway responses in asthma. *Curr Allergy Asthma Rep* 2006;6:112–6. [PubMed: 16566860]
- Springer T, Galfre G, Secher DS, Milstein C. Mac-1: a macrophage differentiation antigen identified by monoclonal antibody. *Eur J Immunol* 1979;9:301–6. [PubMed: 89034]
- Tomkinson A, Duez C, Cieslewicz G, Pratt JC, Joetham A, Shanafelt MC, Gundel R, Gelfand EW. A murine IL-4 receptor antagonist that inhibits IL-4- and IL-13-induced responses prevents antigen-induced airway eosinophilia and airway hyperresponsiveness. *J Immunol* 2001;166:5792–800. [PubMed: 11313423]
- Umetsu DT, DeKruyff RH. The regulation of allergy and asthma. *Immunol Rev* 2006;212:238–55. [PubMed: 16903918]
- Webb DC, Cai Y, Mattheai KI, Foster PS. Comparative roles of IL-4, IL-13, and IL-4R α in dendritic cell maturation and CD4⁺ Th2 cell function. *J Immunol* 2007;178:219–27. [PubMed: 17182558]
- Wright SD, Weitz JI, Huang AJ, Levin SM, Silverstein SC, Loike JD. Complement receptor type three (CD11b/CD18) of human polymorphonuclear leukocytes recognizes fibrinogen. *Proc Natl Acad Sci U S A* 1988;85:7734–8. [PubMed: 2971974]

Zhu J, Yamane H, Cote-Sierra J, Guo L, Paul WE. GATA-3 promotes Th2 responses through three different mechanisms: induction of Th2 cytokine production, selective growth of Th2 cells and inhibition of Th1 cell-specific factors. *Cell Res* 2006;16:3–10. [PubMed: 16467870]

C

D

E

F

G **Figure 1.**

Multicolor FACS analysis of cell types that are increased in OVA-challenged mice compared to PBS-controls. **(A)** Conventional analysis cellular infiltrate include histological analyses of lung tissue sections stained with standard H & E techniques. Scalebars represent 100 μ m.

(B) Cytocentrifugation and differential staining of BAL allows identification of eosinophils, lymphocytes, and other cell types. Cells in BAL of OVA-challenged mice showed particularly high numbers of eosinophils, which exhibited circularized nuclei. **(C–E)** In contrast, FACS-

based analyses allowed simultaneous identification of seven different cell types based on surface markers. Examples are included for lung cells analyzed from mice that were unstained **(C)** or stained lung cells from mice challenged with PBS **(D)** or OVA **(E)**. Fluorescence

intensity for isotype-control antibodies did not change in between PBS- and OVA-challenged lung cells (data not shown). Live cells were distinguished from debris using a marker gate for which all cells located on the right-hand side of the vertical line were considered live cells. A

polygonal gate was constructed in the FSC vs. SSC dot plot to identify those cells with size and granularity properties corresponding to lymphocytes. These cells appear as red dots in all dot plots. Polygonal gates were constructed for each surface marker and relative numbers of cells falling within those gates are shown in the lower right for each set of lung cells. **(F)** Percent

of each cell type measured in 30,000 cells was calculated for mice challenged with either PBS **(white bars; N = 8)** or OVA **(black bars; N = 9)**. Results represent mean + SE and statistical significance was determined by student's t test (* $p < 0.05$). **(G)** Propidium iodide was used to

membrane permeability, i.e. dead cells.

Figure 2. B220^{hi} cells consist of two subpopulations B220^{hi}CD11b⁻ and B220^{hi}CD11b⁺, both of which increase in OVA-challenged mice. **(A)** Histograms display lung cells from PBS-challenged mice in top row and OVA-challenged mice in the lower row. A marker gate was used to identify B220^{lo} and B220^{hi} cells. The latter were then plotted in a dot plot diagram to distinguish and quantify those B220^{hi} cells negative and positive for CD11b. **(B)** Number of cells falling within B220^{hi}CD11b⁻ and B220^{hi}CD11b⁺ were calculated for mice challenged with PBS (**white bars**; N = 8) or OVA (**black bars**; N = 9). Results represent mean + SE and statistical significance was determined by student's t test (*p < 0.05).

Figure 3.

Analysis of the heterogeneous population of CD11b⁺ cells shows multiple subpopulations. **(A)** A gate was constructed for CD11b⁺ cells and differences examples are shown for unstained cells, PBS-challenged lung cells, and OVA-challenged lung cells. **(B)** Dot plots are shown for each cell type to determine which cell type contain subpopulations coexpressing CD11b. Nearly all cell types contained a subset falling within the CD11b gate, but with varying SSC properties. **(C)** Gates were constructed for CD11b⁺ cells exhibiting either SSC^{lo} or SSC^{hi} properties and cells from PBS- or OVA-challenged mice were analyzed for percent of lung cells falling within each gate. **(D)** Evaluation of lung cells from mice challenged with PBS (white bars; N = 8) or OVA (black bars; N = 9). Results represent mean + SE and statistical significance was determined by student's t test (*p < 0.05).

Figure 4.

Lung cells sorted for CD4 and CD11b can be analyzed for mRNA levels for factors involved in inflammation and Th2 immune responses. Cells falling within gates for CD4 or CD11b markers were sorted into complete media. Cells sorted from two mice challenged with PBS (**white bars**) were pooled and cells sorted from two mice challenged with OVA (**black bars**) were pooled. Cells were pelleted by centrifugation, total RNA was extracted, cDNA synthesized, and real-time PCR performed to determine levels of target genes relative to the housekeeping mRNA, hprt. Results are shown for GATA-3 mRNA levels in CD4⁺ cells and IL-1 β levels in CD11b⁺ cells. Results represent mean + SE and statistical significance was determined by student's t test (* $p < 0.05$).