Identification of Myeloid Cell Subsets in Murine Lungs Using Flow Cytometry

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Although the antibody-based recognition of cell-surface markers has been widely used for the identification of immune cells, overlap in the expression of markers by different cell types and the inconsistent use of antibody panels have resulted in a lack of clearly defined signatures for myeloid cell subsets. We developed a 10-fluorochrome flow cytometry panel for the identification and quantitation of myeloid cells in the lungs, including pulmonary monocytes, myeloid dendritic cells, alveolar and interstitial macrophages, and neutrophils. After the initial sorting of viable CD45⁺ leukocytes, we detected three leukocyte subpopulations based on CD68 expression: CD68⁻, CD68^{low}, and CD68^{hi}. Further characterization of the CD68^{hi} population revealed CD45⁺/CD68^{hi}/F4/80⁺/CD11b⁻/CD11c⁺/Gr1⁻ alveolar macrophages and CD45⁺/CD68^{hi}/F4/80⁻/CD11c⁺/Gr1⁻/CD103⁺/major histocompatibility complex (MHC) class II^{hi} dendritic cells. The CD68^{low} population contained primarily CD45⁺/CD68^{low}/F4/80⁺/CD11b⁺/CD11c⁺/Gr1⁻/CD14^{low} interstitial macrophages and CD45⁺/CD68^{low}/F4/80⁺/CD11b⁺/CD11c⁻/Gr1^{low}/CD14^{hi} monocytes, whereas the CD68⁻ population contained neutrophils (CD45⁺/CD68⁻/F4/80⁻/CD11b⁺/Gr1^{hi}). The validity of cellular signatures was confirmed by a morphological analysis of FACS-sorted cells, functional studies, and the depletion of specific macrophage subpopulations using liposomal clodronate. We believe our approach provides an accurate and reproducible method for the isolation, quantification, and characterization of myeloid cell subsets in the lungs, which may be useful for studying the roles of myeloid cells during various pathological processes.

Keywords: interstitial macrophages; alveolar macrophages; monocytes; myeloid dendritic cells; neutrophils

The heterogeneous population of myeloid cells in the lungs is important for maintaining homeostasis and regulating inflammation, injury, and remodeling. These cells are thought to originate from bone marrow-derived precursors, and they differentiate into mature cells with a variety of functional properties, depending on environmental cues. Multiple studies have demonstrated functional and phenotypic differences between myeloid

CLINICAL RELEVANCE

We recognized a need for this work because studies of lung myeloid cells to date have been limited by their inconsistent use of cellular markers and a lack of clearly defined signatures, resulting in difficulties comparing studies from different laboratories and generalizing findings across different model systems. These issues led us to undertake a study to identify and quantify myeloid cell populations comprehensively in the lungs, using flow cytometry.

cell subsets, including monocytes, myeloid dendritic cells, macrophages, and neutrophils. However, the specific identification and quantification of subsets of these cells in tissues are complex, and a comprehensive scheme for isolating these cells from the lungs is not readily available. To date, studies of lung myeloid cells have been limited by the inconsistent use of cellular markers and a lack of clearly defined signatures for these cells, resulting in difficulties comparing studies from different laboratories and generalizing findings across different model systems. Therefore, we undertook a study to identify and quantify myeloid cell populations comprehensively in the lungs, using flow cytometry.

Based on the recent explosion of information regarding the mechanisms that underpin innate immune responses, a clear need is apparent for better strategies to identify and study myeloid cell subpopulations in the lungs. The traditional method for the identification and quantification of lung myeloid cells involves the manual counting of cells obtained from the airways by bronchoalveolar lavage (BAL). This methodology normally yields a predominance of alveolar macrophages, because these are the primary immune cells in the airspaces. Interstitial macrophages, however, comprise a substantial portion of the lung myeloid cell population (1), and may have different functional properties compared with alveolar macrophages. Interstitial macrophages, together with pulmonary monocytes and dendritic cells, are not readily obtained by BAL. In addition to manual counting, myeloid cell subsets can be identified to some extent by the immunohistochemical examination of lung sections, which allows for analyses based on the anatomical locations and expressions of specific markers. Unfortunately, the expression of some commonly used markers is not restricted to myeloid cells, and others may overlap in different myeloid cell subsets (2–5). As a result, the immunohistochemical identification of lung myeloid subpopulations may be not sufficiently specific. Moreover, immunohistochemical evaluation is not sensitive enough to resolve differences in the level of expression among

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particular antigens within myeloid cell subsets, which may be necessary for optimal phenotypic identification.

Flow cytometry offers advantages over other methodologies for detecting the expression of multiple cellular determinants in a single staining protocol, together with assessments of expression levels. Despite increasing data on the expression of different cellular antigens by myeloid cell subsets, information about the coexpression of multiple markers in distinguishing specific cell subpopulations remains limited and controversial. For example, whether the expression of CD68 is restricted to macrophages or can be ascertained in dendritic cells and monocytes remains debatable (5–7). Similarly, F4/80 has been reported on the surface of most (but not all) macrophages, resident and inflammatory monocyte subsets, and even myeloid dendritic cells in the spleen (2). In some studies, CD11c is reported to be expressed in lung dendritic cells, macrophages, and pulmonary monocytes (3, 8). In addition to differences in the fluorochromes and antibodies used, part of the explanation for conflicting results in the literature relates to differences in controls, gating strategies, and methods to account for macrophage autofluorescence.

With the goal of developing a robust and reproducible approach to identify myeloid cell subpopulations in the lungs, we evaluated specific cellular signatures associated with these cells. We tested and validated a 10-fluorophore flow cytometry panel for the identification of myeloid cell subsets in the lungs, including alveolar and interstitial macrophages, dendritic cells, monocytes, and neutrophils. Overall, our approach should provide a more accurate and reproducible means to isolate, quantify, and characterize myeloid cell subsets in normal lungs and during different pathological processes, such as infection and lung injury.

MATERIALS AND METHODS

Animal Experiments

All animal care and experimental procedures were approved and performed according to guidelines issued by the Institutional Animal Care and Use Committee at Vanderbilt University. Eight- to 10-week-old mice (FVB or C57BL6/J background) and OT-II transgenic mice (C57BL6/J background; Jackson Laboratories, Bar Harbor, ME) were used in these experiments.

Isolation of Lung Cells

To obtain resident alveolar macrophages, BAL was performed by instilling four 0.8-ml aliquots of sterile normal saline through a tracheotomy tube. To obtain single-cell suspensions, lungs were perfused with sterile PBS, removed *en bloc*, and digested as described elsewhere (9).

Flow Cytometry and FACS

Single-cell suspensions were incubated with a Fc receptor block ($1 \mu\text{g}/1 \times 10^6$ cells; BD Bioscience, San Diego, CA) to reduce nonspecific antibody binding. The panel of antibodies used in these experiments included CD45-APC-Cy7 (clone 30-F11), CD11b-V450 or PE-Cy7 (clone M1/70), CD86-PE-Cy7 (clone GL1), Gr1-PerCP-Cy5.5 (clone RB6-8C5), and streptavidin-V500 (all from BD Bioscience); CD68-FITC (clone FA-11) and CD204-Alexa Fluor 648 (clone 2F8) from AbD Serotec (Raleigh, NC); CD14-PE-Cy7 (clone Sa14-2), CD103-APC (clone 2E7), and CD206-APC (clone C068C2) from Biolegend (San Diego, CA); F4/80-PE (clone BM8) from Invitrogen (Carlsbad, CA); and CD11c-PE-Cy5 (clone N418) and MHCII-biotin (clone NIMR-4) from e-Bioscience (San Diego, CA). Dead cells were excluded using a Live/Dead Fixable Blue Dead Cell Stain Kit (Invitrogen) or 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) (Sigma-Aldrich, St. Louis, MO). Flow cytometry was performed using BD LSR II and BD FACS Aria III flow cytometers (BD Bioscience), and data were analyzed with FlowJo software (TreeStar, Ashland, OR). For morphological characterization, cells were sorted by FACS, loaded onto slides using a Cytospin 2 (Shandon Southern Products, Runcorn, Cheshire, United Kingdom),

and stained using a modified Wright formula (American Scientific Products, McGaw Park, IL).

Antigen-Specific Stimulation of T-Lymphocytes *Ex Vivo*

Splenic CD4⁺ T cells were isolated from OT-II mice using a CD4⁺ T-cell isolation kit (Miltenyi Biotec, Auburn, CA). OT-II transgenic mice express the murine α -chain and β -chain T-cell receptor that pairs with the CD4 coreceptor, and is specific for chicken ovalbumin 323–339. To obtain myeloid cell subsets, leukocytes from the lungs of wild-type (WT; C57BL6/J) mice were enriched using magnetic murine CD45 microbeads (Miltenyi Biotec) before FACS for the isolation of different myeloid cell populations. After pretreatment with $1 \mu\text{g}/\text{ml}$ of OT-II-specific peptide (ovalbumin [OVA]_{323–339}–ISQAVHAAHAEINEAGR) for 24 hours, myeloid cell subsets were washed three times with PBS, and 10,000 cells per well were added in coculture with carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeled OT-II spleen CD4⁺ T lymphocytes (1×10^5 cells/well) in a 96-well plate. After 4 days of coculture, the proliferation of OT-II CD4⁺ T cells was analyzed by measuring CFSE intensity via flow cytometry.

Depletion of Macrophages with Clodronate

Clodronate (dichloromethylene diphosphonic acid; Sigma-Aldrich) or sterile PBS-containing liposomes were prepared as described elsewhere (10). For the depletion of alveolar macrophages, mice were anesthetized and intubated with $100 \mu\text{l}$ of liposomal clodronate, as described elsewhere (9). For the depletion of interstitial macrophages, mice received $200 \mu\text{l}$ of liposomal clodronate through tail-vein (IV) injection, as previously described (11).

Statistical Analysis

Data were analyzed using GraphPad Prism version 5.0 software (GraphPad Software, Inc., San Diego, CA), and values are presented as means $\pm$ SEMs. Comparisons between animal groups were performed using one-way ANOVA with the Tukey *post hoc* test. $P < 0.05$ was considered statistically significant.

RESULTS

Characterization of Alveolar Macrophages and Myeloid Dendritic Cells by Flow Cytometry

To identify specific myeloid cell subsets in the lungs, we began by sorting viable cells based on CD45 expression. Although our protocol for generating single-cell suspensions from lung tissue results in some nonviable cells, these dead cells are primarily CD45-negative (nonimmune). After evaluating several approaches to separate the leukocyte population into different subsets, we settled on an approach based on the utilization of CD68, which has been widely applied as a pan-macrophage marker. We detected three leukocyte subpopulations in the lungs of adult mice, based on CD68 expression: CD68-negative (CD68[−]), CD68^{low}, and CD68^{hi} (Figure 1A). In normal adult mice, alveolar macrophages (AMs) account for the vast majority of cells obtained by BAL (12). Because this population is readily available for study, we began by characterizing AMs. We assessed the expression of CD68 in cells collected by BAL, and found that more than 80% of viable CD45⁺ leukocytes in the BAL were detected in the CD68^{hi} gate (Figure 1B). We also evaluated CD68 expression in the remaining lung cells after BAL, and found a reduced proportion of CD68^{hi} cells in lung tissue after BAL. To characterize AMs further, we tested other markers previously associated with these cells, including CD11b, CD11c, and F4/80 within the CD68^{hi} myeloid cell population in the BAL. As shown in Figure 1C, the vast majority of CD68^{hi} cells in the BAL were negative for CD11b. However, approximately 80% of these cells were positive for both F4/80 and CD11c (Figure 1D). Importantly, we chose our gates for CD11b, CD11c, and F4/80 staining based on negative

Figure 1. Separation of myeloid cell subsets in the lungs according to CD68 expression. (A) Flow cytometry plots and (B) an analysis of the percentage of CD68⁺, CD68^{low}, and CD68^{hi} leukocytes obtained by bronchoalveolar lavage (BAL) and in single-cell suspensions from whole lungs. Before the analysis of CD68 expression, cells were sorted based on viability (live cells have a limited uptake of viability dye) and CD45 expression. FSC-A and SSC-A indicate forward and side scatter, respectively. Representative plots demonstrate (C) the expression of CD11b and F4/80, and (D) the expression of CD11c and F4/80 within viable CD45⁺/CD68^{hi} leukocytes in the BAL. The negative control consisted of a sample stained with viability dye and CD45 and CD68 antibodies (no F4/80, CD11b, and CD11c antibodies were added). The data are presented as the means $\pm$ SEMs of three mice per group for entire lungs or lungs after lavage analysis. For the analysis of BAL leukocytes, cells from six mice were pooled together for analysis. The data represent the mean of two pooled samples.

controls for cells in the CD68^{hi} gate in which cells were selected, based on viability, CD45 staining, and CD68 staining, but without the other antibodies. This approach allowed us to differentiate autofluorescence from true immunostaining.

Next, we evaluated CD68^{hi} myelocytes obtained from single-cell suspensions of lung tissue. Similar to BAL cells, the majority of CD68^{hi} myelocytes in whole lungs were positive for CD11c and F4/80 (Figure 2A, *gate R2*), and negative for CD11b (Figure 2B). In addition, CD68^{hi} myelocytes were essentially all negative for Gr1 (Figure 2C), which has been used primarily as a marker for neutrophils and immature myeloid cells. Based on these studies, we identified AMs as CD45⁺/CD68^{hi}/F4/80⁺/CD11b⁻/CD11c⁺/Gr1⁻.

By investigating CD68^{hi} cells further in lung tissue, we detected a population of CD45⁺/CD68^{hi}/CD11c⁺ leukocytes that were negative for F4/80 (Figure 2A, *gate R1*). These cells expressed higher concentrations of MHCII compared with AMs (Figure 2G). Previously, two major subsets of CD11c⁺/MHCII^{hi} lung dendritic cells (DCs) were reported, based on CD103 expression (13, 14). Therefore, we analyzed the expression of CD103 leukocytes within the R1 gate, and found that more than 80% of cells within the R1 gate expressed MHCII and CD103 (Figure 2H). Together, this information suggests that CD45⁺/CD68^{hi}/F4/80⁻/CD11c⁺/Gr1⁻/MHCII^{hi} cells represent myeloid DCs, predominantly the CD103⁺ subset.

Identification of Interstitial Macrophages, Monocytes, and Polymorphonuclear Cells in the Lungs

After identifying AMs and DCs in the CD68^{hi} leukocyte population, we turned our attention to the CD68^{low} subset. Because

CD68^{low} cells appeared to be more prominent in lung tissue compared with BAL, we wondered whether cells in the CD68^{low} gate would include interstitial macrophages (IMs) and monocytes. To define the appropriate gating for additional markers in the CD68^{low} cell population, we used negative controls in which cells were selected based on viability, CD45 staining, and CD68 staining, but without the other antibodies (as already described). From the CD68^{low} (and CD68⁻) leukocyte gates, we found that autofluorescence in each additional channel (including those used for CD11c, CD11b, and F4/80 staining) was lower in comparison to the negative control from the CD68^{hi} compartment (in Figures 2D and 2E, *dashed lines* represent gating extrapolated from the negative control sample from the CD68^{hi} population). This approach of using different negative control gatings for the CD68^{low} and CD68^{hi} cells allowed us to detect low-level positive staining that would not have been apparent if we had used negative controls for the entire CD68 (or CD45) gate, because this includes the highly autofluorescent CD68^{hi} AMs. Based on this strategy, we found that approximately 65% of cells in the CD68^{low} gate were positive for F4/80, and approximately 50% were positive for CD11c (Figures 2D and E). In addition, a large majority of CD68^{low} lung leukocytes (> 90%) were CD11b⁺ (Figure 2E). However, the level of expression for F4/80 and CD11c by CD68^{low} leukocytes was lower compared with the CD68^{hi} population.

To characterize CD68^{low} lung leukocytes further, we analyzed the expression of Gr1 by these cells. Anti-Gr1 antibodies (which recognize both Ly6-G and Ly6-C antigens) identify a heterogeneous population of myeloid cells, including mature and immature

Figure 2. Identification of myeloid cell subsets in the lungs by FACS. Flow cytometry plots demonstrate (A) the expression of CD11c and F4/80, (B) the expression of CD11b and F4/80, and (C) the expression of CD11b and Gr1 within viable CD45⁺/CD68^{hi} leukocytes in entire lungs from naive (untreated) mice. The negative control consisted of viable CD45⁺/CD68^{hi} lung leukocytes with no F4/80, CD11b, CD11c, and Gr1 antibodies added. Flow cytometry plots demonstrate (D) the expression of CD11c and F4/80, (E) the expression of CD11b and F4/80, and (F) the expression of CD11b and Gr1 within viable CD45⁺/CD68^{low} or CD45⁺/CD68⁻ leukocytes in entire lungs. The negative control consisted of viable CD45⁺/CD68^{-/low} lung leukocytes with no F4/80, CD11b, CD11c, and Gr1 antibodies added. Dashed lines represent the gating extrapolated from negative control samples from the CD68^{hi} population. (G and H) Expression of MHCII and CD103 within viable CD45⁺/CD68^{hi}/F4/80⁻/CD11c⁺ myeloid dendritic cells (DCs; R1 gate) or CD45⁺/CD68^{hi}/F4/80⁺/CD11c⁺ alveolar macrophages (AMs; R2 gate). The negative control (cont) consisted of viable CD45⁺/CD68^{hi} leukocytes (with no MHCII antibodies added). (I) Expression of F4/80 and (J) expression of CD11c within viable CD45⁺/CD68^{low}/CD11b⁺/Gr1⁻ interstitial macrophages (IMs; gate R3) or viable CD45⁺/CD68^{low}/CD11b⁺/Gr1^{low} pulmonary monocytes (gate R4). The negative control consisted of viable CD45⁺/CD68^{low} lung leukocytes with no F4/80 or CD11c antibodies added. Each experiment was repeated at least three times.

polymorphonuclear cells (PMNs) that express high concentrations of Gr1, and monocytes that are reported to express low concentrations of Gr1 (15, 16). By assessing the expression of Gr1 and CD11b within the CD68^{low} gate, we identified distinct populations of CD11b⁺ cells that were Gr1⁻ (Figure 2F, gate R3) or that expressed low concentrations of the marker (Figure 2F, gate R4).

These findings suggested that CD45⁺/CD68^{low}/CD11b⁺/Gr1⁻ leukocytes could represent IMs, whereas CD45⁺/CD68^{low}/CD11b⁺/Gr1^{low} cells could represent pulmonary monocytes. Interestingly, we found that both of these leukocyte subsets expressed low concentrations of F4/80 (Figure 2I), but only CD45⁺/CD68^{low}/CD11b⁺/Gr1⁻ cells expressed CD11c (Figure 2J).

Investigation of the viable $CD45^+/CD68^-$ leukocyte gate revealed cells that were positive for CD11b and expressed high concentrations of Gr1 (Figure 2F, *gate R5*). These cells were negative for F4/80. This combination of markers suggested that this cell population ($CD45^+/CD68^-/F4/80^-/CD11b^+/Gr1^{hi}$) could be identified as PMNs.

To confirm the identification of myeloid cell subsets by flow cytometry, we performed experiments to determine the morphology of these cell populations. We sorted lung leukocytes by FACS, loaded them onto slides, and analyzed cell morphology after Wright staining. $CD45^+/CD68^{hi}$ leukocytes demonstrated an appearance consistent with AMs (Figure 3A, *gate R2*). $CD45^+/CD68^-/CD11b^+/Gr1^{hi}$ cells showed the characteristic polymorphonuclear appearance of neutrophils (*gate R5*). An examination of $CD45^+/CD68^{low}/CD11b^+/Gr1^{low}$ lung leukocytes (*gate R4*) revealed that the majority of cells had a kidney-shaped nucleus, which is a common morphologic appearance of monocytes. We also found that the majority of $CD45^+/CD68^{low}/CD11b^+/Gr1^-$ cells (*gate R3*) were of a size similar to that of monocytes, but with oval nuclei and less developed cytoplasm than cells identified as AMs, consistent with previous descriptions of IMs (17). To confirm differences further between monocytes and IMs, we used CD14, because this marker has been shown to be highly expressed in monocytes (8, 18). We evaluated the expression of CD14 within the cell populations identified as pulmonary monocytes and IMs, as well as monocytes isolated from blood. We found that cells we had identified as pulmonary monocytes ($CD45^+/CD68^{low}/CD11b^+/Gr1^{low}$ cells) and blood monocytes expressed CD14 at similar concentrations (Figure 3B). In contrast, cells identified as IMs ($CD45^+/CD68^{low}/CD11b^+/Gr1^-$) demonstrated a lower level of CD14 expression. Together, these additional findings support our scheme for the classification of individual subpopulations of myeloid cells in the lungs.

Use of Liposomal Clodronate to Delete Subpopulations of Myeloid Cells in the Lungs

Previous studies suggested that an intratracheal (IT) injection of liposomes containing clodronate depletes AMs, whereas an IV instillation of clodronate primarily eliminates IMs (11, 19, 20). Therefore, in the present study, we used these approaches to investigate myeloid cell depletion after IT or IV clodronate, and

to confirm the association of cellular signatures as already described with AMs and IMs subsets. For these experiments, WT FVB mice received a single IT or IV injection of liposomal clodronate. Three days later, single-cell suspensions from lung tissue were analyzed using flow cytometry. We found that an IT injection of clodronate significantly reduced the population of AMs (Figure 4A). Further analysis revealed that this decrease was mostly attributable to the elimination of $F4/80^+/CD11c^+$ cells (Figure 4B). Notably, the reduction in $CD45^+/CD68^{hi}/F4/80^+/CD11c^+$ AMs after IT clodronate treatment was accompanied by an increase in the proportion of $CD45^+/CD68^{hi}/F4/80^-/CD11c^+$ cells ($2.2 \pm 0.02 \times 10^5$ in AMs versus $1.7 \pm 0.01 \times 10^5$ in control cells), which we have defined as $CD103^+$ DCs. IT clodronate did not significantly affect the IM population.

In contrast to these findings, IV clodronate injection caused a marked reduction in $CD68^{low}/CD11b^+$ myelocytes (Figure 4C). Further detailed analysis demonstrated that this reduction occurred specifically within the IM population ($CD68^{low}/CD11b^+/Gr1^-$). The percentage of $CD11b^+/Gr1^{low}$ pulmonary monocytes within the $CD68^{low}$ gate was substantially increased after IV clodronate (Figure 4D). However, no differences were observed in absolute numbers of monocytes ($1.9 \pm 0.02 \times 10^5$ in monocytes versus $1.3 \pm 0.01 \times 10^5$ in control cells). Interestingly, IV clodronate reduced the total number of AMs, but this effect was substantially less than the reduction observed after IT clodronate. Neither IT nor IV clodronate changed the number of PMNs in the lungs (data not shown).

Analysis of Antigen-Dependent T-Cell Proliferation Induced by Lung Myeloid Cell Subsets

To characterize cells identified as DCs, AMs, and IMs further using our protocol, we investigated the ability of these myeloid cell subsets to stimulate the antigen-specific proliferation of T-lymphocytes *ex vivo*. Staining with CD68 antibodies requires the permeabilization of cells, thus limiting the use of this strategy for functional studies. Therefore, to isolate lung myeloid cell subsets, we enriched for $CD45^+$ pulmonary leukocytes using microbeads, and then sorted viable (DAPI-negative) myelocyte subsets based on their differential expression of F4/80 and CD11c. As shown in Figure 5A, AMs were recognized as $F4/80^+/CD11c^{hi}$ cells, whereas DCs were identified as $CD11c^{hi}$ leukocytes that were negative for F4/80. Although monocytes

Figure 3. Morphological appearance of myeloid cell subsets in the lungs, and differentiation between interstitial macrophages and monocytes. (A) Representative plots illustrate the gating strategy for the FACS sorting of lung myeloid cells, and microphotographs demonstrate the cell morphology of $CD45^+/CD68^{hi}$ alveolar macrophages (*gate R2*), $CD45^+/CD68^-/CD11b^+/Gr1^{hi}$ lung neutrophils (*gate R5*), $CD45^+/CD68^{low}/CD11b^+/Gr1^{low}$ pulmonary monocytes (*gate R4*), and $CD45^+/CD68^{low}/CD11b^+/Gr1^-$ interstitial macrophages (*gate R3*) isolated from the lungs of naive (untreated) FVB mice. (B) Expression of CD14 within $CD45^+/CD68^{low}/CD11b^+/Gr1^{low}$ lung monocytes (from *gate R4*), $CD45^+/CD68^{low}/CD11b^+/Gr1^-$ interstitial macrophages (interstitial MΦ, from *gate R3*), and monocytes obtained from peripheral blood. Bars: 50 μ m. The negative control consisted of samples stained with viability dye and CD45, CD68, CD11b, and Gr1 antibodies (no CD14 antibodies were added).

Figure 4. The depletion of lung macrophages using intra-tracheal (IT) or intravenous (IV) injections of liposomal clodronate reduces the AM and IM populations, respectively. (A) The number of total CD68^{hi}/F4/80⁺ AMs and (B) the percentage of CD45⁺/CD68^{hi} leukocytes expressing F4/80 and CD11c in the lungs of FVB mice on Day 3 after an IV or IT injection of clodronate or control (PBS) liposomes. (C) The number of total CD45⁺/CD68^{low}/CD11b⁺ myeloid cells and (D) the percentage of CD45⁺/CD68^{low}/CD11b⁺/Gr1^{low} monocytes or CD45⁺/CD68^{low}/CD11b⁺/Gr1⁺ IMs in lungs of FVB mice on Day 3 after an IV or IT injection of clodronate or control (PBS) liposomes. Data are presented as the means $\pm$ SEMs of three mice per group. * $P < 0.05$, compared with control samples. The experiment was repeated at least twice.

and IMs were also identified as cells expressing F4/80, we found that these cells differentially expressed CD11c. Although cells identified as IMs expressed low concentrations of CD11c,

monocytes were identified as cells negative for CD11c. Our conclusion was supported by the observation that although both subsets expressed CD11b, only F4/80⁺/CD11c⁻ monocytes

Figure 5. Analysis of antigen-specific stimulation of T cells by lung myeloid cells *ex vivo*. (A) Strategy for FACS sorting of viable (negative for DAPI) myeloid dendritic cells (DCs), alveolar macrophages (AMs), interstitial macrophages (IMs), and monocytes (Mon) from murine lungs and (B) the expression of CD68 by these leukocyte subsets. Before FACS, lung leukocytes were enriched using murine CD45 magnetic microbeads. (C) Expression of CD11b and Gr1 by FACS-sorted pulmonary monocytes (Mon) and interstitial macrophages (IM). (D) Proliferation of CFSE-labeled OT-II splenic CD4⁺ T lymphocytes (1 $\times$ 10⁵ cells) after 4 days of coculture with lung myeloid DCs, AMs, or IMs loaded with OT-II-specific ovalbumin 323-339 peptide. Contr, control.

were positive for Gr1 (Figure 5C). We also confirmed that DCs, AMs, IMs, and monocytes sorted from lung tissue using this strategy expressed the expected concentrations of CD68 (Figure 5B). To test whether FACS-sorted AMs, DCs, and IMs are efficient at stimulating antigen-specific CD4⁺ T-cell proliferation, we pretreated these cells with OVA_{323–339} peptide, followed by coculture with CFSE-stained OT-II CD4⁺ T cells. We found that the cells identified as myeloid DCs induced the strongest T-cell proliferation response, IMs induced a much lower level of T-cell proliferation, and AMs exerted a minimal effect on the antigen-specific proliferation of OT-II CD4⁺ T cells (Figure 5D).

Expression of Polarization Markers by Myeloid Cell Subsets in the Lungs

The surface receptors CD204 (scavenger receptor A) and CD206 (mannose receptor) function to enhance the phagocytic properties of macrophages, and may indicate an alternative (M2) polarization of these cells (21). In contrast, the expression of the costimulatory molecule CD86 and of MHCII has been associated with the classic (M1) polarization of macrophages (21, 22). To phenotype myeloid cells further, we assessed the expression of these markers in AMs, IMs, pulmonary monocytes, and myeloid DCs in single-cell suspensions from lung tissue. We found that CD45⁺/CD68^{hi}/F4/80⁺/CD11b⁺/CD11c⁺ AMs represented the major source of CD204 and CD206 expression (Figures 6A and 6B). Although CD45⁺/CD68^{hi}/F4/80⁺/CD11c⁺/CD103⁺ myeloid DCs also expressed these markers, the level of expression was significantly lower in comparison with AMs. Interestingly, whereas CD45⁺/CD68^{low}/CD11b⁺/Gr1⁺ IMs expressed CD204, these cells did not express CD206. Both AMs and myeloid DCs demonstrated higher CD86 expression compared with IMs and monocytes, but any substantial expression of MHCII was only observed in myeloid DCs (Figures 6C and 6D). IMs expressed very little CD86 and MHCII, and pulmonary monocytes were essentially negative for all four markers. These findings are consistent with the concept that DCs are poor phagocytes but efficient antigen-presenting cells, whereas AMs represent the major phagocytic population in the lung but are not efficient in T-cell activation. Despite the lack of polarizing stimuli, AMs display both M1 and M2 markers in the basal state.

DISCUSSION

We established specific cellular signatures and a strategy for the identification of myeloid cell subsets in murine lungs, including AMs, IMs, monocytes, myeloid DCs, and PMNs (Figure 7). We found that all of these cells except PMNs express CD68, but the level of expression in these cells helped to discriminate between subsets of myeloid cells. Whereas AMs and myeloid DCs were both found to express high concentrations of CD68, AMs expressed F4/80 and CD11c, but were negative for CD11b. In contrast, myeloid DCs were negative for F4/80, and most expressed CD103. AMs also expressed higher concentrations of CD204 and CD206 than other myeloid populations in the lungs, and both AMs and myeloid DCs expressed the costimulatory marker CD86. Monocytes and IMs were identified as cells expressing low concentrations of CD68. These cells were positive for CD11b and F4/80. Despite similarities, monocytes and IMs can be distinguished according to their expression of CD11c, CD14, and Gr1. PMNs were recognized as cells that were negative for CD68 and positive for CD11b, and that expressed high concentrations of Gr1. Together, these experiments identify specific sets of markers that are characteristic of different myeloid cell subsets. This information should provide a more accurate and reproducible method for isolating and quantifying myeloid cells in the lungs.

Figure 6. Characterization of CD204, CD206, CD86, and MHCII expression by myeloid cells. Flow cytometry plots demonstrate (A) the expression of CD204 (scavenger receptor A), (B) the expression of CD206 (mannose receptor), (C) the expression of CD86, and (D) the expression of MHCII in CD45⁺/CD68^{low}/CD11b⁺/Gr1^{low} lung monocytes (Mon), CD45⁺/CD68^{low}/CD11b⁺/Gr1⁺ IMs, CD45⁺/CD68^{hi}/F4/80⁺/CD11b⁺/CD11c⁺ AMs, and CD45⁺/CD68^{hi}/F4/80⁺/CD11b⁺/CD11c⁺ myeloid DCs in single-cell suspensions from lungs ($n = 5$ mice per group). * $P < 0.05$, compared with lung monocytes. ** $P < 0.05$, compared with all myeloid cell subsets. MFI, mean fluorescence intensity. The negative control consisted of samples stained with viability dye and CD45 and CD68 antibodies (CD86, MHCII, CD204, or CD206 antibodies were not added).

The glycoprotein CD68 (also known as macrophage marker) has been reported by a number of groups as a specific pan-macrophage marker. Consistent with this notion (23), we established that lung

Figure 7. Representation of markers associated with myeloid cell subsets in murine lungs. AM, alveolar macrophages; IM, interstitial macrophages; mDC, myeloid dendritic cells; Mon, monocytes; PMNs, polymorphonuclear cells (neutrophils).

myeloid cells other than neutrophils are CD68-positive. Although CD68 was previously reported to be expressed in both AMs and IMs (24), we confirmed and expanded these data by demonstrating that AMs and IMs express different concentrations of CD68. This observation was supported by several experimental findings. In addition to the finding that macrophages obtained by BAL were CD68^{hi}, the removal of cells by BAL reduced the proportion of cells remaining in the lung within the CD68^{hi} subset. CD68^{hi} lung leukocytes isolated by FACS sorting exhibited the size and morphologic characteristics of AMs. Moreover, in agreement with previous observations suggesting that an IT instillation of liposomal clodronate specifically depletes AMs (11, 20, 25), we observed a substantial reduction of CD68^{hi} cells after IT clodronate injection. In contrast, an IV injection of clodronate predominantly reduced the CD68^{low} leukocyte population. These data support the conclusion that lung leukocytes expressing low concentrations of CD68 represent IMs. Interestingly, we found that IV clodronate also partially reduced the number of CD68^{hi} AMs. Bowden and Adamson previously demonstrated that the pool of IMs may contribute to the maintenance of the AM population (26, 27). Moreover, Landsman and Jung described how AMs predominantly originate from monocyte-derived IMs, rather than differentiate directly from blood monocytes (28). Therefore, we speculate that the small reduction in CD68^{hi}-expressing cells observed after IV clodronate was attributable to the compromised differentiation of new AMs from the reduced pool of CD68^{low} IMs, and not to the direct clodronate-induced apoptosis of AMs.

Although AMs and IMs can be identified according to their level of expression of CD68, this marker can also be expressed by other cells, including DCs and monocytes (5, 29, 30). Similarly, other markers widely used in the analysis of macrophages, including F4/80, CD11b, and CD11c, are not restricted to macrophages. The glycoprotein F4/80 is thought to be a relatively specific cell-surface marker for murine macrophages. However, it can be expressed by eosinophils as well as macrophages (31, 32). In lung tissue, we found that IMs and monocytes expressed this antigen weakly. The expression of CD11b (alternatively known also as Mac-1 α) has been proposed to be macrophage-specific, and has been used by many groups for the isolation of macrophages from different organs, including the lungs. However, we found that this marker is not expressed by most AMs, which is consistent with some previous findings (33). We also found that lung macrophages express CD11c, the marker originally associated with DCs (24, 34, 35). Thus, lung macrophages, and particularly AMs, may be phenotypically distinct from macrophages in other organs. In addition, our data indicate that

a single surface marker cannot reliably identify all myeloid cell subsets in the lungs.

As opposed to single markers, combinations of markers may represent specific signatures associated with different subsets of lung myeloid cells (4, 35, 36). AMs, which comprise the most studied myeloid cell subset in the lung, have been identified by some groups as cells expressing CD11c and distinguishable from myeloid DCs by their high autofluorescence, low level of expression of MHCII, and lack of expression of CD11b (28, 34). Bedoret and colleagues proposed to define AMs as CD11c⁺ cells expressing CD68 and F4/80, whereas myeloid DCs were reported as CD68⁻/F4/80⁻/CD11c⁺ (24). Although we found that AMs and myeloid DCs exhibit differential F4/80 and MHCII expression, both cell types expressed CD68 in our experiment. In 2007, Sung and colleagues identified lung myeloid DCs as either CD11b⁻/CD103⁺ or CD11b⁺/CD103⁻ cells (13). In agreement with this categorization, we ascertained that approximately 85–90% of cells we identified as DCs (CD68^{hi}/F4/80⁻/CD11c⁺/MHCII^{hi}) expressed either CD11b or CD103. On the other hand, we found that AMs were negative for both CD11b and CD103. Our scheme for the separation of DCs and macrophages was supported by functional studies involving antigen-dependent T-cell proliferation. In addition, the depletion of macrophages by IT or IV clodronate revealed a reduction in AMs and IMs, but not in cells identified as myeloid DCs. Our findings are in agreement with previous observations by Jakubzick and colleagues, who observed an increase in the proportion of myeloid DCs after the depletion of AMs (37).

Our characterization of IMs differs from that in some previous reports. We established that IMs express F4/80, CD11b, and low concentrations of CD11c. In contrast, others have identified IMs as cells expressing F4/80, but negative for CD11c (24). Our experiments revealed that these F4/80⁺/CD11b⁺/CD11c⁻ cells represent pulmonary monocytes. Functionally, pulmonary IMs differ from AMs based on the capacity of IMs to stimulate antigen-driven T-cell responses (Figure 5D).

Although differences between our findings and those in previous studies can be explained to some extent by the use of different antibodies and fluorochromes, our gating strategy and the use of controls also distinguish our approach. To set gates, we used a modification of the fluorescence minus one (FMO) approach. Although isotype control antibodies are useful for determining antibody specificity, the FMO approach is superior for determining boundaries in multicolor flow, resulting in a more accurate discrimination between positive and negative populations (38). Our approach involved staining cells with all

antibodies in the panel (except the antibodies for which we were controlling), and setting separate boundaries for the CD68^{hi} and CD68^{low/-} populations (which produce a different autofluorescence) in the control gates. Although this method is not as rigorous as the FMO approach, it is more feasible, given the number of leukocytes available in murine lung homogenates. By using this strategy, we were able to set clear negative control gates that allowed for the specific identification of positively staining cell populations.

Macrophages, monocytes, and DCs have been recognized as integral cells regulating the balance between tissue destruction and repair. The capacity of cells to exhibit a variety of phenotypic profiles in response to different stimuli depends on their expression of specific receptors. CD204 (scavenger receptor A) and CD206 (mannose receptor) are cell-surface receptors that function to enhance the phagocytic properties of mononuclear myelocytes, and to facilitate the clearance of harmful particles and pathogens that reach the lower respiratory tract. The activation of these receptors leads to the recognition and internalization of unopsonized pathogens, apoptotic host cells, and modified lipoproteins. Consistent with this idea, we found a predominance of cells expressing CD204 and CD206 within the population of AMs. Although the expression of these markers was also detected in DCs, the level of expression was significantly lower in comparison with AMs. These findings are in agreement with previous reports indicating that myeloid DCs possess poor phagocytic properties (39). At the same time, the high expression of CD86 and MHCII in DCs supports the presence of efficient antigen-presenting properties in these cells.

In conclusion, our study outlines a scheme for the specific identification of different myeloid cell subsets in the lungs of mice, using flow cytometry. An enhanced ability to identify myeloid cell subsets should facilitate the further elucidation of the roles played by this complex, heterogeneous group of cells in the pathogenesis of different lung diseases.

Author disclosures are available with the text of this article at www.atsjournals.org.

References

- Lehnert BE, Valdez YE, Holland LM. Pulmonary macrophages: alveolar and interstitial populations. *Exp Lung Res* 1985;9:177–190.
- Gordon S, Hamann J, Lin HH, Stacey M. F4/80 and the related adhesion-GPCRs. *Eur J Immunol* 2011;41:2472–2476.
- Hume DA. Applications of myeloid-specific promoters in transgenic mice support *in vivo* imaging and functional genomics but do not support the concept of distinct macrophage and dendritic cell lineages or roles in immunity. *J Leukoc Biol* 2011;89:525–538.
- Hashimoto D, Miller J, Merad M. Dendritic cell and macrophage heterogeneity *in vivo*. *Immunity* 2011;35:323–335.
- Song L, Lee C, Schindler C. Deletion of the murine scavenger receptor CD68. *J Lipid Res* 2011;52:1542–1550.
- Lykens JE, Terrell CE, Zoller EE, Divanovic S, Trompette A, Karp CL, Aliberti J, Flick MJ, Jordan MB. Mice with a selective impairment of IFN-gamma signaling in macrophage lineage cells demonstrate the critical role of IFN-gamma-activated macrophages for the control of protozoan parasitic infections *in vivo*. *J Immunol* 2010;184:877–885.
- Paulson KE, Zhu SN, Chen M, Nurmohamed S, Jongstra-Bilen J, Cybulsky MI. Resident intimal dendritic cells accumulate lipid and contribute to the initiation of atherosclerosis. *Circ Res* 2010;106:383–390.
- Gordon S, Taylor PR. Monocyte and macrophage heterogeneity. *Nat Rev Immunol* 2005;5:953–964.
- Zaynagetdinov R, Sherrill TP, Polosukhin VV, Han W, Ausborn JA, McLoed AG, McMahon FB, Gleaves LA, Degryse AL, Stathopoulos GT, et al. A critical role for macrophages in promotion of urethane-induced lung carcinogenesis. *J Immunol* 2011;187:5703–5711.
- Everhart MB, Han W, Parman KS, Polosukhin VV, Zeng H, Sadikot RT, Li B, Yull FE, Christman JW, Blackwell TS. Intratracheal administration of liposomal clodronate accelerates alveolar macrophage reconstitution following fetal liver transplantation. *J Leukoc Biol* 2005;77:173–180.
- Thepen T, Van Rooijen N, Kraal G. Alveolar macrophage elimination *in vivo* is associated with an increase in pulmonary immune response in mice. *J Exp Med* 1989;170:499–509.
- Lohmann-Matthes ML, Steinmuller C, Franke-Ullmann G. Pulmonary macrophages. *Eur Respir J* 1994;7:1678–1689.
- Sung SS, Fu SM, Rose CE Jr, Gaskin F, Ju ST, Beatty SR. A major lung CD103 (alphaE)-beta7 integrin-positive epithelial dendritic cell population expressing langerin and tight junction proteins. *J Immunol* 2006;176:2161–2172.
- Geurtsvan Kessel CH, Lambrecht BN. Division of labor between dendritic cell subsets of the lung. *Mucosal Immunol* 2008;1:442–450.
- Gabrilovich DI, Nagaraj S. Myeloid-derived suppressor cells as regulators of the immune system. *Nat Rev Immunol* 2009;9:162–174.
- Strauss-Ayali D, Conrad SM, Mosser DM. Monocyte subpopulations and their differentiation patterns during infection. *J Leukoc Biol* 2007;82:244–252.
- Dethloff LA, Lehnert BE. Pulmonary interstitial macrophages: isolation and flow cytometric comparisons with alveolar macrophages and blood monocytes. *J Leukoc Biol* 1988;43:80–90.
- Mathai SK, Gulati M, Peng X, Russell TR, Shaw AC, Rubinowitz AN, Murray LA, Siner JM, Antin-Ozerkis DE, Montgomery RR, et al. Circulating monocytes from systemic sclerosis patients with interstitial lung disease show an enhanced profibrotic phenotype. *Lab Invest* 2010;90:812–823.
- Koay MA, Gao X, Washington MK, Parman KS, Sadikot RT, Blackwell TS, Christman JW. Macrophages are necessary for maximal nuclear factor-kappa B activation in response to endotoxin. *Am J Respir Cell Mol Biol* 2002;26:572–578.
- Mircescu MM, Lipuma L, Van Rooijen N, Pamer EG, Hohl TM. Essential role for neutrophils but not alveolar macrophages at early time points following *Aspergillus fumigatus* infection. *J Infect Dis* 2009;200:647–656.
- Mantovani A, Sica A, Sozzani S, Allavena P, Vecchi A, Locati M. The chemokine system in diverse forms of macrophage activation and polarization. *Trends Immunol* 2004;25:677–686.
- Mantovani A, Sozzani S, Locati M, Allavena P, Sica A. Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. *Trends Immunol* 2002;23:549–555.
- Daley JM, Thomay AA, Connolly MD, Reichner JS, Albina JE. Use of Ly6G-specific monoclonal antibody to deplete neutrophils in mice. *J Leukoc Biol* 2008;83:64–70.
- Bedoret D, Wallemacq H, Marichal T, Desmet C, Quesada CF, Henry E, Closset R, Dewals B, Thielen C, Gustin P, et al. Lung interstitial macrophages alter dendritic cell functions to prevent airway allergy in mice. *J Clin Invest* 2009;119:3723–3738.
- Holt PG, Oliver J, Bilyk N, McMenamin C, McMenamin PG, Kraal G, Thepen T. Downregulation of the antigen presenting cell function(s) of pulmonary dendritic cells *in vivo* by resident alveolar macrophages. *J Exp Med* 1993;177:397–407.
- Bowden DH, Adamson IY. Role of monocytes and interstitial cells in the generation of alveolar macrophages I: kinetic studies of normal mice. *Lab Invest* 1980;42:511–517.
- Bowden DH, Adamson IY. Alveolar macrophage response to carbon in monocyte-depleted mice. *Am Rev Respir Dis* 1982;126:708–711.
- Landsman L, Jung S. Lung macrophages serve as obligatory intermediate between blood monocytes and alveolar macrophages. *J Immunol* 2007;179:3488–3494.
- Soilleux EJ, Morris LS, Leslie G, Chehimi J, Luo Q, Levroney E, Trowsdale J, Montaner LJ, Doms RW, Weissman D, et al. Constitutive and induced expression of DC-SIGN on dendritic cell and macrophage subpopulations *in situ* and *in vitro*. *J Leukoc Biol* 2002;71:445–457.
- Vermaelen K, Pauwels R. Pulmonary dendritic cells. *Am J Respir Crit Care Med* 2005;172:530–551.
- McGarry MP, Stewart CC. Murine eosinophil granulocytes bind the murine macrophage-monocyte specific monoclonal antibody F4/80. *J Leukoc Biol* 1991;50:471–478.
- Chow A, Lucas D, Hidalgo A, Mendez-Ferrer S, Hashimoto D, Scheiermann C, Battista M, Leboeuf M, Prophete C, Van Rooijen N, et al. Bone marrow CD169⁺ macrophages promote the retention of hematopoietic stem and progenitor cells in the mesenchymal stem cell niche. *J Exp Med* 2011;208:261–271.
- Lin KL, Suzuki Y, Nakano H, Ramsburg E, Gunn MD. CCR2⁺ monocyte-derived dendritic cells and exudate macrophages produce influenza-induced pulmonary immune pathology and mortality. *J Immunol* 2008;180:2562–2572.

34. Vermaelen K, Pauwels R. Accurate and simple discrimination of mouse pulmonary dendritic cell and macrophage populations by flow cytometry: methodology and new insights. *Cytometry A* 2004;61:170–177.
35. Guth AM, Janssen WJ, Bosio CM, Crouch EC, Henson PM, Dow SW. Lung environment determines unique phenotype of alveolar macrophages. *Am J Physiol Lung Cell Mol Physiol* 2009;296:L936–L946.
36. Galli SJ, Borregaard N, Wynn TA. Phenotypic and functional plasticity of cells of innate immunity: macrophages, mast cells and neutrophils. *Nat Immunol* 2011;12:1035–1044.
37. Jakubzick C, Tacke F, Llodra J, Van Rooijen N, Randolph GJ. Modulation of dendritic cell trafficking to and from the airways. *J Immunol* 2006;176:3578–3584.
38. Perfetto SP, Chattopadhyay PK, Roederer M. Seventeen-colour flow cytometry: unravelling the immune system. *Nat Rev Immunol* 2004;4:648–655.
39. Lambrecht BN, Hammad H. The role of dendritic and epithelial cells as master regulators of allergic airway inflammation. *Lancet* 2010;376:835–843.