Flow Cytometry Reveals Similarities Between Lung Macrophages in Humans and Mice

To the Editor:

Findings in murine models implicate subpopulations of alveolar macrophages in the pathogenesis of lung injury and fibrosis; however, the relevance of these findings for humans with chronic lung disease is unknown in part because of a lack of proper tools to identify macrophage heterogeneity in the human lung. Here we report a flow cytometry protocol that allows unambiguous identification of alveolar macrophages, interstitial macrophages, and monocytes in the human lung and in bronchoalveolar lavage fluid. We validated this panel using normal lung tissue and tissue from patients with chronic obstructive pulmonary disease and lung fibrosis. We found evidence of heterogeneity within human alveolar macrophage populations, which suggest parallels between murine and human macrophage development and differentiation.

Lung macrophages are essential for maintaining homeostasis in the healthy lung and play an important role in pulmonary diseases (1). For decades, alveolar macrophages, which are abundant in the alveolar space of the normal lung, were considered a homogenous population of cells derived from and continuously replenished by circulating monocytes originating from the bone marrow. In careful studies of murine lung development, several groups of investigators have overturned this paradigm. They discovered that alveolar macrophages originate from fetal monocytes, which populate the lungs during the first days of life (2–4). These "tissue-resident" alveolar macrophages are capable of self-renewal, and they or their daughter cells persist in the lung over the lifespan of the host (1, 2). In addition, a healthy mouse lung contains a small fraction of the monocyte-derived interstitial macrophages and extravasated and intravascular monocytes (2, 5, 6). Various insults can induce recruitment of the monocytes into the lung. These monocytes transition through "interstitial macrophages" before differentiating into monocyte-derived alveolar macrophages, which may persist in the lung after injury (7). Previously, we found that although these monocyte-derived alveolar macrophages are phenotypically similar to tissue-resident alveolar macrophages, there were small differences in their surface marker expression after injury, suggesting that subtle changes in surface markers might distinguish monocyte-derived from tissue-resident macrophages (8).

Although human macrophages are less well studied, the murine data predict the presence of at least three distinct populations of monocytes or macrophages in the human lung: alveolar macrophages, interstitial macrophages, and monocytes. Using workflows and protocols we developed in mice, we generated a multicolor flow cytometry protocol to unambiguously identify these populations in the human lung. Using these markers we also observed heterogeneity in the alveolar macrophage population, which may reflect the relative contribution of monocyte-derived versus tissue-resident cells to the alveolar macrophage pool. Because this panel relies exclusively on the identification of cell surface markers, it is appropriate for sorting and recovering live cells for subsequent analysis.

All procedures using human tissues were approved by the Northwestern University Institutional Review Board. We obtained biopsies from normal human donor lungs before transplantation (nine samples) and explanted lungs from recipients with chronic obstructive pulmonary disease (five recipients), systemic sclerosis-associated interstitial lung disease (one recipient), mixed connective tissue disease (one recipient), idiopathic lung fibrosis (one recipient), and 10 bronchoalveolar lavage (BAL) samples from patients admitted to the intensive care unit who are being evaluated for pneumonia. We also obtained fixed sections of lung tissue from five stillborn infants. We developed a standardized protocol to process the lungs for flow cytometric analysis (*see* online supplement for details). After screening several markers, we found that HLA-DR, CD169, and CD206 allow accurate separation of the three monocyte/macrophage populations: alveolar macrophages ($CD11b^{+}HLA\text{-}DR^{++}CD206^{++}CD169^{+}$), interstitial macrophages ($CD11b^{+}HLA\text{-}DR^{++}CD206^{+}CD169^{-}$), and monocytes ($CD11b^{+}HLA\text{-}DR^{+}CD206^{-}CD169^{-}$) (Figure 1A and *see* Figure E1 in the online supplement). Interestingly, CD206, which has been suggested to indicate "M2 polarization" of macrophages, is ubiquitously expressed in macrophage populations in the normal human lung (9). Immunofluorescent microscopy confirmed the expected anatomical localization of these cells (Figure 1B). The same populations were identified in the diseased explanted lungs of patients with chronic obstructive pulmonary disease, systemic sclerosis-associated lung fibrosis, and mixed connective tissue disease undergoing lung transplantation, and all key cell markers (HLA-DR, CD169, and CD206) performed well (data not shown). We then screened several markers that might identify heterogeneity

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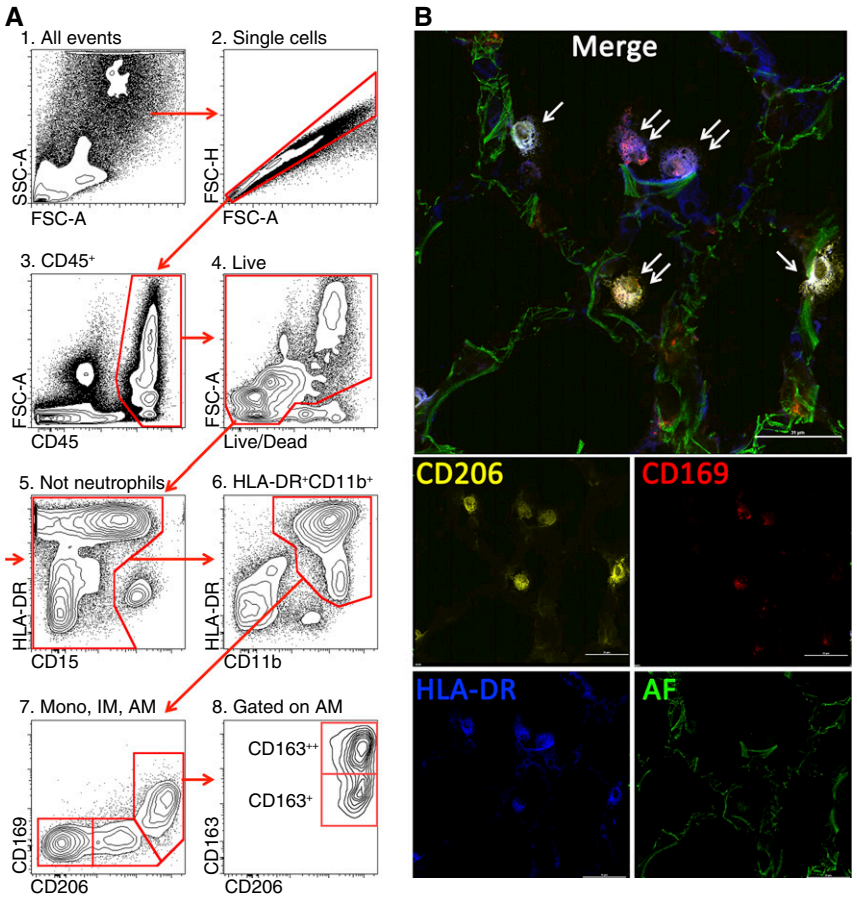

Figure 1. (*A*) Gating strategy used to identify lung monocytes and macrophages. After excluding doublets (*2*), cells of hematopoietic origin were identified as $CD45^+$ (*3*), followed by exclusion of the dead cells (precautions were taken not to gate out highly autofluorescent alveolar macrophages) (*4*). Neutrophils were identified as $CD11b^{++}CD15^+CD16^+HLA\text{-}DR^-$ cells and excluded from analysis (*5*). We then gated on $CD11b^{++}HLA\text{-}DR^+$ cells (*6*). This allows separation from natural killer (NK) cells ($CD11b^+HLA\text{-}DR^-CD56^+$) and highly autofluorescent eosinophils ($CD11b^{+/-}HLA\text{-}DR^-Siglec\ 8^+$). Finally, using CD206 and CD169, cells were separated into three subpopulations: alveolar macrophages ($CD11b^+HLA\text{-}DR^{++}CD206^{++}CD169^+FSChighSSChigh$, AM), interstitial macrophages ($CD11b^+HLA\text{-}DR^{++}CD206^+CD169^-$, IM), and monocytes ($CD11b^+HLA\text{-}DR^+CD206^-CD169^-$, mono) (*7*). CD163 identifies two subpopulations of alveolar macrophages (*8*). (*B*) Immunofluorescent microscopy on a normal human lung. *Orange* is CD206 PE, *red* is CD169 AF647, *blue* is HLA-DR BV421, and *green* is autofluorescence in fluorescein isothiocyanate channel. *Single arrows* indicate $HLA\text{-}DR^+CD206^+CD169^-$ cells (interstitial macrophages), and *double arrows* indicate $HLA\text{-}DR^+CD206^+CD169^+$ cells (alveolar macrophages). *Scale bar* is 31 μm.

in the alveolar macrophage population. We found that the alveolar macrophage population in all studied samples (from both normal and diseased lungs) can be further subdivided into $CD163^{++}$ and $CD163^+$ subpopulations (Figure 1A, *panel 8*). CD163 is a scavenger receptor for hemoglobin/haptoglobin complex highly expressed on tissue macrophages, and it has been proposed as a marker of "resolving" monocyte-derived macrophages (10, 11). Because our previous studies in a mouse model of lung fibrosis demonstrated that differential expression of Siglec F allows discrimination of the tissue-resident and monocyte-derived macrophages (8), we now speculate that the heterogeneity in CD163 expression in human alveolar macrophages may also reflect their differential ontogeny. Alternatively, heterogeneity in CD163 may reflect a different activation state and/or anatomical localization; these questions need further evaluation in the large, well-described cohorts of patients. Intriguingly, BAL analysis did not reveal the presence of interstitial macrophages $CD11b^+HLA\text{-}DR^{++}CD206^+CD169^-$, nor did it show CD163 heterogeneity in alveolar macrophages observed in tissue biopsies (Figure E2). These findings parallel findings in mice in which BAL fluid incompletely described the macrophage populations in the normal or injured murine lung (12).

In newborn mice, tissue-resident alveolar macrophages are absent at birth but differentiate from lung fetal monocytes in the first days of life (3). We reasoned that if a similar developmental pathway occurs in humans, monocytes and interstitial macrophages, but not alveolar macrophage markers, should be found in human lungs before birth. Immunostaining of lung sections from stillborn infants were negative for both $CD169^+$ cells and $CD206^+$ cells located in the alveoli, whereas $CD206^+$ cells were present in the interstitium (Figure E3). These results are consistent with earlier work suggesting that alveolar macrophages are not found in stillborn infants in the absence of infection but are routinely found in neonates (13). Although our laboratories are completely independent, our findings, protocol, and conclusions

are similar to those described by Yu and colleagues in this issue of the *Journal* (pp. 13–24)., highlighting the utility and reproducibility of this approach for future studies of alveolar macrophage populations in the human lung (14). ■

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