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In Vivo Characterization of Alveolar and Interstitial Lung Macrophages in Rhesus Macaques: Implications for Understanding Lung Disease in Humans

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Macrophages are phagocytic cells derived from blood monocytes that play important roles in innate immunity and steady-state homeostasis (1). Lung macrophages are highly heterogeneous, based on their anatomical location, specialized function, and activation state (2–4). At least three types of macrophages have been identified in the lung that include alveolar macrophages (AMs), interstitial macrophages (IMs), and intravascular/margined vascular macrophages that differ by location and function (2, 5). AMs are widely accepted to function in removing particles and microorganisms in the alveoli, whereas IMs are believed to function in regulating tissue fibrosis, inflammation, and Ag presentation (2). Margined vascular macrophages appear to function in the cross talk between APCs in the lung interstitium for recruiting neutrophils or myeloid cells (5). The existence of lung macrophage subsets with different functional properties requires additional analyses to better understand their contributions to lung disease pathogenesis.

Macrophages were recognized more than a century ago when Elie Metchnikoff first described phagocytosis and defined a role for these cells in inflammation in the 1880s, but questions remain about monocyte/macrophase heterogeneity (1). This is because macrophage classifications primarily were based on in vitro experiments that did not address the influences of tissue microenvironment where monocytes/macrophages dwell (1). For example, AMs are incapable of developing tolerance to endotoxin (LPS) at levels induced in mononuclear phagocytes or in macrophages located in other tissue compartments such as peritoneal cavity, bone marrow, or spleen. This difference seems to be due to a rich GM-CSF microenvironment in the lung (6). In addition, there appear to exist species-specific responses in expression of functional genes and homologous proteins, as well as unique markers on macrophage populations residing in various tissues and hosts that contribute to variability in macrophage classification schemes (7–9).
to humans, especially in relation to studies about AIDS, tuberculosis (TB) (14), endometriosis (11), and aging (15). Data derived from human tissues have provided the most authentic information about the subsets, functions, and roles of macrophages in lung disease progression. However, limited access to antemortem human tissues restricts studies to better understand lung disease pathogenesis in situ. Rhesus macaques are thus useful as models of human diseases because of their similar physiology (15, 16). Studies related to human lung biology focus primarily on cells recovered from bronchoalveolar lavage (BAL), but macrophage subsets obtained from BAL are primarily AMs and rarely contain IMs (17). The purpose of this study, therefore, was to characterize IMs in lung tissue of rhesus macaques as a model to better understand the biology of human lung macrophages.

Materials and Methods

Animals, BrdU injections, and sample collections

Adult male Indian rhesus macaques (Macaca mulatta) between the ages of 4 and 10 y were used for these studies. All animal procedures were performed according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and were approved by the Tulane University Institution Animal Care and Use Committee. The BrdU (Sigma-Aldrich, St. Louis, MO) was prepared at 30 mg/ml in PBS (pH 7.2, Ca/Mg-free; Mediatech, Manassas, VA) and filter sterilized prior to inoculation i.v. at 60 mg/kg. In some studies, another thymidine analog, 5-ethyl-2′-deoxyuridine (EdU; Molecular Biology, Carlsbad, CA), was prepared at 25 mg/ml in PBS and inoculated i.v. at 50 mg/kg. EDTA-preserved blood specimens were obtained 24 h before and several time points after BrdU injection (i.e., 24, 48, 72, and 96 h) for evaluation of monocyte turnover rates. BAL specimens were obtained by bronchoscopy 48 h and/or 7 d later as indicated. At necropsy, lung tissue (∼3 cm³) was obtained 48 h after BrdU injection.

Isolation of immune system cells from lung

Lung tissues were digested in medium containing 200 U/ml type IV collagenase (cat. no. 4189; Worthington Biochemical, Lakewood, NJ) and 0.05 mg/ml DNAase I (cat. no.10104159001; Roche Applied Science, Indianapolis, IN) to obtain single-cell suspensions as described in the online data (Supplemental Fig. 1A). Classically, BAL has been used to recover AMs, and these cells also could be recovered by lung wash (agitation) of tissues digested during lung resection surgery (18) as diagrammed in Supplemental Fig. 1A. AMs were obtained via BAL using bronchoscopy or by lung wash from 2-cm³ sections of lung tissue from which visible bronchi were removed, followed by three rinses with 30 ml RPMI 1640 (Cellgro, Manassas, VA) and filter sterilized prior to inoculation i.v. at 60 mg/kg. In some studies, another thymidine analog, 5-ethyl-2′-deoxyuridine (EdU; Molecular Biology, Carlsbad, CA), was prepared at 25 mg/ml in PBS and inoculated i.v. at 50 mg/kg. EDTA-preserved blood specimens were obtained 24 h before and several time points after BrdU injection (i.e., 24, 48, and 72 h) for evaluation of monocyte turnover rates. BAL specimens were obtained by bronchoscopy 48 h and/or 7 d later as indicated. At necropsy, lung tissue (∼3 cm³) was obtained 48 h after BrdU injection.

Flow cytometry and data analysis

Two hundred microliters whole-blood or 10⁵ BAL cells were stained for flow cytometry as previously described (19). Abs used for immunofluorescence staining were anti–CD163 (1:20; Clone 10D6; catalog no. CD163-L-U; Leica Biosystems Newcastle, Newcastle, U.K.), anti-CD206 (also named anti–MRC1; 1:100; Clone: polyclonal; catalog no. HPA004114; Sigma-Aldrich, St. Louis, MO), anti–Caveolin-1 (1:100; Clone: polyclonal; catalog no. C4490; Sigma-Aldrich), and anti-BrdU (1:50; Clone: BU1/75; catalog no. nb500-169; Novus Biologicals, Littleton, CO). Apoptotic cells were detected with the Click-iT TUNEL Alexa Fluor 647 Imaging Assay kit (catalog no. C10247; Invitrogen) based on the manufacturer’s protocol. DNase-treated slides were used as positive controls and those treated with DNase but without EdU in the reaction were used as negative controls in the TUNEL assay.

Imaging was performed with a Leica TCS SP2 confocal microscope equipped with three lasers (Leica Microsystems) under oil immersion (×40,×63,×100), and a resolution of 512 × 512 pixels. Adobe Photoshop software (version 7.0; Adobe Systems) was used to process and assemble the images.

Classical macrophage activation signaling ex vivo and intracellular cytokines staining

Single cells isolated from rhesus macaque lung tissue were adjusted to 5 × 10⁶ live cells/ml RPMI 1640 (Cellgro, Manassas, VA) containing 10% FCS (catalog no. 26140-079; Life Technologies, Grand Island, NY) and 100 IU/ml penicillin/streptomycin (EMD Millpore, Billerica, MA) and filter sterilized prior to inoculation i.v. at 30 mg/ml in PBS. After 3 h incubation at 37 °C and 5% CO₂, nonadherent cells were removed by washing with 1 ml PBS. Adherent cells were then supplied with 1 ml fresh complete medium, medium containing 100 ng/ml recombinant human IFN-γ (catalog no. RIFGNG100; Thermo Scientific, Rockford, IL), or medium with IFN-γ plus 100 ng/ml Escherichia coli serotype 0127:B8 LPS. After 4-h stimulation, cells were harvested and stained with surface markers CD3-V500, CD8-V500, CD20-V500, and these cells also could be recovered by lung wash (agitation) of tissues digested during lung resection surgery (18) as diagrammed in Supplemental Fig. 1A. AMs were obtained via BAL using bronchoscopy or by lung wash from 2-cm³ sections of lung tissue from which visible bronchi were removed, followed by three rinses with 30 ml RPMI 1640 (Cellgro, Manassas, VA) and filter sterilized prior to inoculation i.v. at 60 mg/kg. In some studies, another thymidine analog, 5-ethyl-2′-deoxyuridine (EdU; Molecular Biology, Carlsbad, CA), was prepared at 25 mg/ml in PBS and inoculated i.v. at 50 mg/kg. EDTA-preserved blood specimens were obtained 24 h before and several time points after BrdU injection (i.e., 24, 48, and 72 h) for evaluation of monocyte turnover rates. BAL specimens were obtained by bronchoscopy 48 h and/or 7 d later as indicated. At necropsy, lung tissue (∼3 cm³) was obtained 48 h after BrdU injection.

Differential staining of BAL cells

Cytospins were prepared from 200,000 BALs or 50,000 sorted cells by centrifugation (Shandon Cytospin 3; Thermo Electron Corporation) at 400 × g for 3 min, and slides were stained with Wright-Giemsa. Differential counting was performed under light microscopy at ×200 magnification.
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HLA-DRhi, CD11bint, scavenger receptor CD163, and mannose receptor CD206 as indicated in Fig. 1B (P1). Epithelial cells, in contrast, exhibited low autofluorescence as shown in Fig. 1B (P2). Moreover, the level of CD45 expression was used to confirm and discriminate epithelial cells from macrophages/lymphocytes (data not shown). Small lymphocytes, B cells, and cell debris were HLA-DR− CD11b− and belonged to the population of cells indicated in Fig. 1B (P3). Because AMs (P1) seemed to comprise at least two subpopulations, we sorted by FACS into AM-H (CD206hiCD163hi) and AM-L (CD206intCD163int), and further analyzed by Wright–Giemsa staining. No morphological differences were observed except that AM-H were larger (higher forward scatter) and more granular (higher side scatter) than AM-L (Supplemental Fig. 2).

Macrophages are the predominant population of immune system cells in normal lung tissue

Previous reports indicated that at least two populations of macrophages (AMs and IMs) have been identified in lung tissue of humans and mice (4). However, BAL samples contained only AMs (Fig. 1A, 1B). To determine the proportion of lung AMs and IMs in rhesus macaques, cell populations isolated from sections of ~2 cm³ from various anatomical sites of lung tissue in relation to areas indicated on a silicone rubber cast of a normal monkey lung (Supplemental Fig. 1B) were analyzed by flow cytometry. The results demonstrated high similarity in expression levels of HLA-DR and CD11b on cells isolated from the different regions of the lung, suggesting a relatively homogenous distribution of immune response cells throughout normal rhesus macaque lung tissue (Supplemental Fig. 1C). Therefore, we performed the remaining analyses with lung cells obtained from area R1 (Supplemental Fig. 1B). From the detailed flow cytometry analysis, CD163 was selected to define lung macrophages in rhesus macaques (22–24). The composition of the cell subsets identified from whole-lung tissue by flow cytometry comprised three populations of myeloid cells as shown in Fig. 2A: granulocytes (HLA-DR− , CD11b+), AMs (HLA-DRhi, CD11bint, CD163+, CD206+), and IMs (HLA-DRhi, CD11bhi, CD163+). Two populations of dendritic cells also were identified that included myeloid dendritic cells (HLA-DRint, CD11bint, CD11c+) and plasmacytoid dendritic cells (HLA-DRint, CD11bdim, CD123+). Four populations of lymphocytes were identified including NK cells, CD4+ T cells, CD8+ T cells, and B cells. The cell subset distribution was characterized in whole-lung tissues from five rhesus macaques, and ~55 and 10% of these were AMs and IMs, respectively (Fig. 2B).

Spatial distribution of AMs and IMs in lung tissue

To corroborate the flow cytometry assessment of lung tissue, we performed immunohistochemistry and confocal imaging to determine the spatial distribution of the AMs and IMs using Ab panels: Panel I included BrdU-FITC, CD163-PE, CD14-ECD, CD4–PCP-Cy5.5, HLA-DR-PE-Cy7, CD206-allophycocyanin, CD11b-AL700, CD16-allophycocyanin-H7, CD3-Pacific blue, CD20-eFluor 450, and CD8-V500; Panel II contained BrdU-FITC, CD163-PE, CD14-ECD, CD123–PCP–Cy5.5, HLA-DR-PE-Cy7, CD11c-allophycocyanin, CD11b-AL700, CD206-allophycocyanin-Cy7, CD3-Pacific blue, CD20-eFluor 450, CD16-V500, and CD8-QD655. Staining for the scavenger receptor CD163 (22) and mannose receptor CD206 (25) were applied to detect the macrophages. Although CD68 (pan-macrophage marker) also stained the same macrophage subsets as CD163 Ab, we used CD163 as a pan-macrophage marker to achieve a better staining profile in lung tissue cells. In addition, Caveolin-1 was used to detect the endothelial cells to discriminate from intravascular cells. Consistent with the results from flow cytometry of BAL and lung tissue digests, CD163+, CD206+ AMs were located almost exclusively in the alveoli of the lung and were larger than IMs (Fig. 3A, 3D). CD163+ CD206−/dim IMs were located in the interstitial spaces of the lung tissue (Fig. 3A–C). IMs were more frequently observed in the peribronchovascular and subpleural regions of the lung (Fig. 3B, 3C, 3E, 3F). The ratios of AM to IM from two monkeys were 3.07 and 4.45, respectively (Table I), corroborating the flow cytometry data presented in Fig. 2B.

High turnover of IMs, but not AMs, during steady-state homeostasis

To evaluate potential functional differences and developmental relationships among blood monocytes (CD14+), IMs, and AMs, we examined expression of 28 cellular markers (Supplemental Table IB) on immunological cells of whole-lung tissue. The phenotype of IMs resembled monocytes in their expression of...
CD14, Mac387, CD11b, HLA-DR, CD68, CD163, CD31, CD64, TLR2, TLR9, CD209, and CD95. Blood monocytes could be distinguished by the expression of CCR2 (receptor for monocyte chemotactic protein-1) that was not expressed on IMs or tissue macrophages (Fig. 4). Moreover, monocytes could be divided into CD36hi and CD36 low subpopulations, but AMs and IMs both expressed high levels of CD36 (Fig. 4). These phenotype relationships suggest that blood monocytes could be the direct precursor of IMs. In addition, AMs were easily distinguished from IMs and monocytes by the expression of CD206 and high expression level of CD11c molecule (Fig. 4).

Incorporation of the thymidine analog, BrdU or EdU, into cellular DNA during the S-phase of the cell cycle is considered a specific marker for dividing cells and is used to track cell migration and differentiation in vivo (19). We reported that detection of BrdU incorporation in blood monocytes over a period of 24 h was a good indicator for monocyte emigration into the blood from bone marrow (monocyte turnover) (19). Therefore, it was expected that BrdU incorporation by tissue macrophages derived from blood monocytes would be detectable after 24 h or later. To better characterize the relationship between blood monocytes, IM, and AM, we injected BrdU i.v. and collected blood specimens 24

FIGURE 2. Macrophages are predominant cells of the immune system in healthy lung. (A) Gating strategy for analyzing cells isolated from lung tissue of healthy rhesus macaques. CD11b+ staining cells were considered to be of myeloid lineage. Granulocytes (CD11b-, HLA-DR-+) were separated after first excluding lymphocytes that stained with CD3/20/8. Then HLA-DRhi, CD11b+, CD163+, CD206+ cells were defined as AMs. IMs were identified as HLA-DRhi, CD11bhi, CD163+ CD206-cells. Myeloid dendritic cells were identified as CD11c+, HLA-DR+, CD163-, CD206-, and plasmacytoid dendritic cells were identified as CD123+, HLA-DR+, CD163-, CD206-. Lymphocytes were small cells (SSCl) and were further divided into CD3/20hi cells including CD4+ T cells, CD8+ T cells, and B cells (CD4-, CD8-, HLA-DR+), whereas CD3/20lo cells comprised CD8+, CD16+ NK cells and CD8+, CD16- NK cells. A small subset of cells not identified with these markers was labeled UN (unidentified cells). The mean percent values (± SD) of each cell population were determined from lung tissues of five rhesus macaques as shown in (B).
and 48 h later. Lung tissues were obtained at necropsy 48 h after BrdU injection. Single-cell suspensions were prepared for analysis of IMs and AMs. More than 35% of IMs and monocytes stained BrdU+, but only 1.518% (±0.24) of AMs were stained BrdU+ 48 h later (Fig. 5A, 5B). These data suggest that IMs originate from blood monocytes and exhibit a relatively short life span during steady-state homeostasis. To verify that IMs differentiate from monocytes rather than self-renew within the lung, IMs were isolated 24 h after BrdU injection and were observed to stain negative for BrdU (data not shown). Moreover, AMs in BAL samples obtained 1, 2, 7, 21, and 28 d after BrdU injection exhibited only marginal levels of staining for BrdU throughout these time points (Supplemental Fig. 3), further corroborating that AMs exhibit a slower turnover rate and appear to be longer-lived cells in the lung alveolar spaces during steady-state homeostasis.

To determine whether apoptosis serves as a feedback mechanism to regulate macrophage population size, we applied TUNEL to assess apoptosis of AMs and IMs in lung tissues of four animals. Results in Fig. 5C and 5D demonstrated that 22.41% (±4.01) of IMs labeled with TUNEL, which was significantly higher than the 5.50% (±1.29) of AMs that labeled with TUNEL (p = 0.007). This suggests that apoptosis represents a potential mechanism for regulating numbers of IMs in the lungs of rhesus macaques during steady-state.

### Monocytes/IMs are precursors to AMs

Our data clearly indicated that in steady-state, AMs are longer-lived cells with negligible cell turnover. It was previously reported that macrophages respond rapidly to tissue injury after exposure to LPS (26) or Streptococcus pneumonia (27) via accelerated recruitment of monocytes to the alveolar spaces. To further characterize this, we performed a BAL procedure and measured the kinetics of AMs in the alveolar space. BrdU (or EdU) was injected followed by BAL 2 d after BrdU injection and again 7 d later (i.e., 5 d after the first BAL) to determine the changes in the alveolar spaces. As shown in Fig. 6A, 18.12% (±1.54) of AMs recovered from BAL 5 d after the initial BAL removal of AMs exhibited staining for BrdU. This was significantly higher than the 2.64% (±0.42) of BrdU-labeled AMs during steady-state homeostasis on day 2 (p < 0.0001), as shown in Fig. 6B. These results suggested an increased influx of AMs either directly from monocytes or from IM (Fig. 6A). Confocal microscopy further demonstrated the transition of an IM from lung tissue to the alveolar space that exhibits an intermediate phenotype marker (increasing the expression of CD206), as well as increasing size (Fig. 6C).

### Table I. Ratio of AMs/IMs in normal lung tissue sections

<table>
<thead>
<tr>
<th>Animal ID</th>
<th>Alveolar</th>
<th>Interstitial</th>
<th>Peribronchovascular</th>
<th>Subpleural</th>
<th>Total AMs</th>
<th>Total IMs</th>
<th>AMs/IMs</th>
</tr>
</thead>
<tbody>
<tr>
<td>GI53</td>
<td>511</td>
<td>14</td>
<td>0</td>
<td>0</td>
<td>88</td>
<td>39</td>
<td>525</td>
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<td>28</td>
<td>0</td>
<td>0</td>
<td>122</td>
<td>77</td>
<td>1163</td>
</tr>
</tbody>
</table>

Observations were recorded from 20 fields per slide under ×200 magnification at 1 slide per monkey.
vivo IFN-γ plus LPS treatment (1.552 ± 0.251-fold, n = 3; p = 0.0886; Fig. 7B). Conversely, the expression of TNF-α in IMs increased significantly after ex vivo IFN-γ plus LPS stimulation (4.822 ± 1.268, n = 3; p = 0.430; Fig. 7B). A priming signal with IFN-γ only failed to induce a significant increased expression of TNF-α in either IMs or AMs (Fig. 7B).

Discussion
Characterizing the biology of all lung macrophage subsets in healthy rhesus macaques is important and as a basis to better understand pulmonary disease pathogenesis during AIDS, TB, and other inflammatory diseases in humans. The results in this study demonstrated that although AMs are the predominant immune cells in the lung, IMs may represent a macrophage subset with different functional properties that are also involved in daily homeostasis and protection against continuous pathogen exposure from the environment. This is suggested by findings that IMs maintain a relatively higher turnover rate, are shorter-lived in steady-state than are AMs, and ex vivo IFN-γ plus LPS treatment significantly increased intracellular expression of TNF-α in IMs but not in AMs. In contrast, AMs exhibited relatively lower turnover during steady-state homeostasis than did IMs or blood monocytes. In

![Figure 4](http://www.jimmunol.org/)

**FIGURE 4.** Phenotype differences between AMs, IMs, and monocytes. Blood monocytes, IMs, and AMs were stained with Abs (Supplemental Table IA) for flow cytometry analyses. Black lines represent isotype control Ab staining, and the filled gray lines indicate specific Ab staining. Histograms are representative of at least three healthy rhesus macaques. Results demonstrate that monocytes, IMs, and AMs could be discerned from each other, but monocytes and IMs were relatively similar to each other.
addition, AMs were reported to be longer-lived cells in lungs of mice (26, 28) and humans (29).

IMs in lung tissues of rhesus macaques resembled blood monocytes phenotypically and were CD11b+, HLA-DR+, Mac387+, CD163+, CD14+, and CD206<sup>2</sup>. In steady-state, IMs exhibited higher turnover rates that were slightly lower than that of blood monocytes, suggesting that monocytes are the direct precursor of the lung IMs. Moreover, the IM population size appeared to be regulated by apoptosis as indicated by high TUNEL+ IMs (22.41%±4.010%).

AMs are relatively static (30) under normal “resting” conditions in mice, but they undergo apoptosis and replacement by IMs after exposure to LPS (26) or Streptococcus pneumonia (27). Consistent with these reports, we demonstrated that after a BAL procedure, there also occurred a rapid differentiation of IM or blood monocyte to AM in the alveoli that may have been induced either by the mechanical nonimmunological removal of AMs and/or by induction of mild inflammation. Moreover, AMs were easily distinguished from IMs and blood monocytes by the expression of the mannose receptor CD206 and heterogeneous CD206 expression among AMs suggests CD206 may be a maturation marker.

**FIGURE 5.** IMs, but not AMs, exhibit high turnover during steady-state homeostasis. Cell turnover was reflected by measuring the uptake of BrdU by AMs, IMs, or monocytes in relation to the total population. As shown in (A), BrdU staining was highest in monocytes and IMs 48 h after BrdU injection and was low in AMs after 48 h and in monocytes after 24 h (n = 4). Staining 24 h after BrdU injection was determined to be a good measure for the production of monocytes and emigration into the blood from bone marrow, so this time point was used to define monocyte turnover (19). Confocal microscopy in (B) confirmed that high turnover of IM and negligible turnover of AM occurs during steady-state based on triple-label confocal microscopy staining for CD163 (red), CD206 (green), and BrdU (that identifies recently arrived cells, blue). Asterisks indicate CD163<sup>−</sup>CD206<sup>−</sup> AMs with no BrdU staining. Arrows indicate CD163 single-positive IMs stained with BrdU. This experiment was performed using samples collected from four different uninfected monkeys necropsied 2 d after BrdU injection. Results in (C) indicated that a significantly higher percent of IMs than AMs were undergoing apoptosis as measured by TUNEL staining. A confocal microscopy image in (D) shows apoptotic (blue), CD163<sup>+</sup> (green) macrophages in normal lung tissue and demonstrates high turnover of IMs in relation to increased apoptosis. Arrows indicate CD163 single-positive IMs stained with TUNEL. Asterisks indicate AMs (CD163<sup>−</sup>) with no TUNEL staining. Confocal images were acquired under an oil objective (×63, fluotar/NA 1.0) and are representative of studies from four monkeys (A, C). *p < 0.05, **p < 0.01, and ***p < 0.0001 (A, C).

**FIGURE 6.** Monocytes/IMs are precursors to AMs. Rhesus macaques were injected with BrdU or EdU nucleic acid analogs and stained for uptake and macrophage markers as indicated. In the first experiment, BAL was performed on day 2 to remove AMs and again on day 7 (i.e., 5 d later) to follow the turnover of the AMs that were repopulating the alveolar space. Results in (A) (n = 8) demonstrated significant increases in repopulating AM turnover 5 d after mechanical removal of AMs via BAL. If no initial BAL is performed on day 2, AM turnover was observed to remain low on day 7 as shown in (B) (n = 6). A macrophage undergoing transition from IMs to AMs is shown in (C) and exhibits expression of macrophage markers CD68 (blue), CD206 (green), and CD163 (red). The image was captured under oil immersion (×63, fluotar/NA 1.0). ***p < 0.0001 (A).
from three uninfected monkeys. Results in (A) demonstrated the gating strategy for AMs and IMs, as well as representative histograms for the intracellular staining of TNF-α expression in AMs and IMs after stimulation from three uninfected monkeys. Results in (B) show the mean fold-change (± SD) in intracellular expression of TNF-α in AMs and IMs after LPS and IFN-γ treatment compared with untreated or IFN-γ alone controls (n = 3). *p < 0.05 in (B).

These findings suggest that IMs are derived from blood monocytes and can serve as intermediates for differentiation into AMs in primates as previously described in mice (31). It is still possible that the bronchoalveolar lavage procedure might have induced mild inflammation and recruitment of blood monocyte to the lung tissue as also described previously (30). Although S. Jung et al. (32) and M. Merad et al. (33) recently used fate mapping techniques to demonstrate that resident murine AMs can proliferate locally for self-maintenance under steady homeostasis, we were not able to directly demonstrate the self-renewal of AMs in macaques. We did not observe BrdU+ AMs and IMs at 24 h after BrdU injection when BrdU+ monocytes were detected in the blood. The appearance of BrdU+ blood monocytes, however, always preceded the appearance of BrdU+ IMs in this study, suggesting a chronological sequence of differentiation from monocytes to IMs. Furthermore, we observed no increased incorporation of BrdU+ by AMs after 48 h, 7 d, 21 d, or 28 d (Supplemental Fig. 3) after BrdU injection. The genetic distance between mice and nonhuman primates also may explain this discrepancy. For example, the human homolog of F4/80, a classical murine macrophage marker, is an epidermal growth factor–like molecule containing a mucin-like receptor 1 that, in humans, is exclusively expressed on eosinophils rather than monocytes/macrophages (8), further supporting the value of studies in nonhuman primates for translating results to humans (34). It is also possible that AMs of rhesus macaques may self-renew, as occurs in mice, but the rate of AM turnover would be much slower because of the low rate of BrdU incorporation observed in this study.

Perhaps more importantly, the higher turnover of IMs and negligible replacement of AMs during steady-state strongly argues that the continuous availability of IMs in lung tissue is required to maintain and reestablish homeostasis. The significant increase in TNF-α production in IMs, but not AMs, in response to IFN-γ plus LPS classical macrophage activation stimuli ex vivo provides additional support that IMs and AMs represent different macrophage subsets with different functional properties in the lung and emphasizes the importance in studying IMs and AMs independently in a physiologically relevant model to better understand pathogenesis of pulmonary diseases such as interstitial lung diseases where inflammatory and immune responses may not be reflected in BAL specimens (35, 36). It is well-known that cells in human BAL specimens fail to reflect the cellular components in the lung interstitium (37), emphasizing the importance to study IMs in addition to AMs to understand the overall macrophage biology of the lung, as well as pathogenesis in macrophage-related lung disease.

Rhesus macaques are phylogenetically similar to humans and provide an excellent model for studying various human diseases (38, 39), suggesting that studies on immune cells in the lung of rhesus macaques are expected to provide important insights about human lung biology. This model is thus expected to be helpful to better understand human lung macrophage characteristics and responses, especially those from different compartments in the lung (including AMs and IMs), and takes advantage of the ability to apply in vivo BrdU labeling to follow cell migration and turnover in situ.

Recently, we demonstrated that high blood monocyte turnover correlates with rapid disease progression to AIDS in SIV-infected rhesus macaques that appeared to result from massive tissue macrophage destruction in mesenteric lymph nodes (19). In this study, we found that IMs exhibited high turnover and apoptosis, and were rapidly replenished with blood monocytes in normal animals, supporting a critical role of IMs in protection against continuous pathogen exposure from the environment. This was also supported by our recent findings that massive SIV infection and destruction of IMs in the SIV-infected macaques correlates with AIDS disease progression and pulmonary tissue damage (Y. Cai, D.X. Liu, M.J. Kuroda, unpublished observations). This rhesus macaque model thus will provide a basis to study human lung macrophages in response to infectious diseases such as SIV/HIV and TB, as well as under noninfectious conditions such as interstitial lung disease, pulmonary hypertension, and chronic obstructive pulmonary disease.

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Disclosures
The authors have no financial conflicts of interest.
References