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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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Alveolar macrophages (AMs) obtained by bronchoalveolar lavage (BAL) are commonly used to study lung macrophage-mediated immune responses. Questions remain, however, about whether AMs fully represent macrophage function in the lung. This study was performed to determine the contribution of interstitial macrophages (IMs) of lung tissue to pulmonary immunity and that are not present in BAL sampling. In vivo BrdU injection was performed to evaluate the kinetics and monocyte/tissue macrophage turnover in Indian rhesus macaques (Macaca mulatta). Lung macrophage phenotype and cell turnover were analyzed by flow cytometry and immunohistochemistry. AMs and IMs in lungs of rhesus macaques composed ∼70% of immune response cells in the lung. AMs represented a larger proportion of macrophages, ∼75–80%, and exhibited minimal turnover. Conversely, IMs exhibited higher turnover rates that were similar to those of blood monocytes during steady-state homeostasis. IMs also exhibited higher staining for TUNEL, suggesting a continuous transition of blood monocytes replacing IMs undergoing apoptosis. Although AMs appear static in steady-state homeostasis, increased influx of new AMs derived from monocytes/IMs was observed after BAL procedure. Moreover, ex vivo IFN-γ plus LPS treatment significantly increased intracellular expression of TNF-α in IMs, but not in AMs. These findings indicate that the longer-lived AMs obtained from BAL may not represent the entire pulmonary spectrum of macrophage responses, and shorter-lived IMs may function as the critical mucosal macrophage subset in the lung that helps to maintain homeostasis and protect against continuous pathogen exposure from the environment.

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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; BioWhittaker, Manassas, VA) and filter sterilized prior to use, and was used to recover AMs and these cells also could be recovered by lung wash (agitation) of tissues recovered 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 containing 5% of FCS and 100 IU/ml penicillin/streptomycin (EMD Millipore, Billerica, MA), and pre-warmed sterile saline. EDTA-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, fluor/NA 1.0) and a resolution of 512 × 512 pixels. Adobe Photoshop software (version 7.0; Adobe Systems) was used to process and assemble the images.

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 recovered 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) containing 10% of FCS (catalog no. 26140-079; Life Technologies) and 100 IU/ml penicillin/streptomycin (EMD Millipore, Billerica, MA). Lung tissues were digested further into ~0.5-mm-thick sections with sterile scalpels, washed at least five times with 30 ml RPMI 1640 containing 5% of FCS and 100 IU/ml penicillin/streptomycin or until the filtrate solution appeared clear, to at least five times with 30 ml RPMI 1640 containing 5% of FCS, 100 IU/ml penicillin/streptomycin, 2 mM L-glutamine (Cellgro, Manassas, VA), 25 mM HEPES (Molecular Biology), 200 U/ml type IV collagenase (catalog no. 4189; Worthington Biochemical, Lakewood, NJ), and 0.05 mg/ml DNAase I (catalog no. 10104159001; Roche Applied Science, Indianapolis, IN) at 37°C for 30 min with orbital shaking at 200 rpm followed by incubation for 10 min at 37°C with intermittent pipetting to further aid tissue digestion. The cell suspension was then subjected to discontinuous density centrifugation over 24% and 50% Percoll (catalog no. 17-0891-01; GE Healthcare, Boston, MA) at 2000 rpm for 20 min (Beckman, Allegra X-12R; Brea, CA). Interstitial cells were recovered from the 24–50% Percoll interface, washed with 2% PBS-FCS (PBS containing 2% FCS), and stored in liquid nitrogen until further analyses.

Flow cytometry and data analysis

Two hundred microliters whole-blood or 10⁵ BAL cells were stained for flow cytometry as previously described to analyze the expression of surface markers and intracellular BrdU or EdU incorporation using the three-laser FACSARia (Becton Dickinson, San Jose, CA) (19). EdU staining was performed using the Click-iT Edu Pacific Blue Flow Cytometry Assay kit (catalog no. C-10418; Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol. Abs used in these analyses are shown in Supplemental Table 1A. Isotype-matched Abs were used to confirm the phenotype of each cell population. Data analyses were performed with FlowJo (Version 9.6; TreeStar) software.

Inmunofluorescence Ab staining and confocal microscopy

Lung tissue sections of 7-μm thickness were treated for immunofluorescence Ab staining as previously described (19). Abs used for immunohistochemistry 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. CI0247; 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, fluor/NA 1.0) 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 × 10⁵ live cells/ml RPMI 1640 (Cellgro, Manassas, VA) containing 10% of FCS (catalog no. 26140-079; Life Technologies) and 100 IU/ml penicillin/streptomycin (EMD Millipore, Billerica, MA) and 0.05 mg/ml DNAase I (catalog no. 10104159001; Roche Applied Science, Indianapolis, IN) to obtain single-cell suspensions as described in the online data Supplemental Fig. 1A. To isolate interstitial cells (Supplemental Fig. 1A), we further digested the remaining lung tissue in 30 ml RPMI 1640 containing 5% of FCS, 100 IU/ml penicillin/streptomycin, 2 mM L-glutamine (Cellgro, Manassas, VA), 25 mM HEPES (Molecular Biology), 200 U/ml type IV collagenase (catalog no. 4189; Worthington Biochemical, Lakewood, NJ), and 0.05 mg/ml DNAase I (catalog no. 10104159001; Roche Applied Science, Indianapolis, IN) at 37°C for 30 min with orbital shaking at 200 rpm followed by incubation for 10 min at 37°C with intermittent pipetting to further aid tissue digestion. The cell suspension was then subjected to discontinuous density centrifugation over 24% and 50% Percoll (catalog no. 17-0891-01; GE Healthcare, Boston, MA) at 2000 rpm for 20 min (Beckman, Allegra X-12R; Brea, CA). Interstitial cells were recovered from the 24–50% Percoll interface, washed with 2% PBS-FCS (PBS containing 2% FCS), and stored in liquid nitrogen until further analyses.

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.

Student t test was used to compare the mean differences between groups using GraphPad Prism version 5.0a for Mac OS Mountain Lion (GraphPad Software, San Diego, CA). A p value <0.05 was considered statistically significant.

Results

AMs comprise the major cell subset recovered in BAL from rhesus macaques

BAL specimens are the most commonly used samples for studying lung macrophages in humans (20), so it is important to characterize macrophages in BAL of rhesus macaques. Healthy rhesus macaques exhibiting no signs of lung disease or infection were used to generate baseline data. By differential counting, >90% of cells from 11 healthy rhesus macaque BAL samples were observed to be AMs (Fig. 1A), which is consistent with results reported on lavage samples from healthy humans (21). Morphological analysis of cells retrieved from FACS revealed that macroque AMs exhibited high autofluorescence because of their large size, as also observed for humans (21). Rhesus AMs expressed...
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 cm² 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, CD11bʰi, CD16³⁺, CD206⁺), and IMs (HLA-DRʰi, CD11bʰi, CD16³⁻). Two populations of myeloid dendritic cells also were identified that included myeloid dendritic cells (HLA-DRʰi, CD11bʰi, CD11c⁻) and plasmacytoid dendritic cells (HLA-DRʰi, CD11bʰi, 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 >5% 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-PeCy7, 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-QDot655. 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 1B) 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 plasmacytid dendritic cells were identified as CD123+, HLA-DR−, CD163+, CD206+. Lymphocytes were small cells (SSC−) and were further divided into CD3/20+ cells including CD4+ T cells, CD8+ T cells, and B cells (CD4−, CD8−, HLA-DR−), whereas CD3/20− 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% (±4.010) of IMs labeled with TUNEL, which was significantly higher than the 5.50% (±1.299) 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% (±1.545) 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% (±0.422) 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).

**Ex vivo macrophage activation signaling with IFN-γ plus LPS significantly increased TNF-α expression in IMs, but not in AMs**

To examine a functional difference between AMs and IMs, we applied ex vivo classical macrophage activation signals, IFN-γ plus LPS, to single lung cell isolates from the lung of rhesus macaque followed by flow cytometry analysis of intracellular TNF-α expression (Fig. 7). The CD206+AMs expressed relatively higher basal levels of TNF-α compared with CD206-IMs (Fig. 7A) in the presence of medium only and increased minimally after ex

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**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>
</tr>
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<tr>
<td></td>
<td>Large</td>
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<tr>
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<td>14</td>
<td>0</td>
<td>44</td>
</tr>
<tr>
<td>EC61</td>
<td>1135</td>
<td>28</td>
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<td>61</td>
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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.

**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+. 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% ± 4.01%).

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 pneumoniae (27). Consistent with these reports, we demonstrated that after a BAL procedure, there 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+CD206+ 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+ (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 IMs (CD163+) 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, fluor/NA 1.0). ***p < 0.0001 (A). the mannose receptor CD206 and heterogeneous CD206 expression among AMs suggests CD206 may be a maturation marker.
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.

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 noninfectious conditions such as interstitial lung disease, pulmonary hypertension, and chronic obstructive pulmonary disease.

FIGURE 7. Ex vivo macrophage activation signaling with IFN-γ plus LPS significantly increased TNF-α expression in IMs, but not in AMs. Adherent single-cell isolates from the lung of uninfected rhesus macaque were stimulated with LPS and IFN-γ ex vivo for 4 h before live/dead and Ab staining for flow cytometry analysis. Results in (A) demonstrated the gating strategy for AMs and IMs, as well as representative histograms for the intracellular staining of TNF-α 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).

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Disclosures
The authors have no financial conflicts of interest.