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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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Abstract

Alveolar macrophages (AM) obtained by bronchoalveolar lavage (BAL) are commonly used to study lung macrophage-mediated immune responses. Questions remain, however, about whether AM fully represent macrophage function in the lung. This study was performed to determine the contribution of interstitial macrophages (IM) 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. AM and IM in lungs of rhesus macaques comprised about 70% of immune response cells in the lung. AM represented a larger proportion of macrophages, approximately 75–80%, and exhibited minimal turnover. Conversely, IM exhibited higher turnover rates that were similar to those of blood monocytes during steady state homeostasis. IM also exhibited higher staining for TdT-mediated dUTP nick end labeling (TUNEL), suggesting a continuous transition of blood monocytes replacing IM undergoing apoptosis. Although AM appear static in steady state homeostasis, increased influx of new AM derived from monocytes/IM was observed following BAL procedure. Moreover, *ex vivo* IFN-γ plus LPS treatment significantly increased intracellular expression of TNF-α in IM but not in AM. These findings indicate that the longer-lived AM obtained from BAL may not represent the entire pulmonary spectrum of macrophage responses, and shorter-lived IM 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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This article has an online data supplement.

Introduction

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 (AM), interstitial macrophages (IM) and intravascular/marginated vascular macrophages that differ by location and function (2, 5). AM are widely accepted to function in removing particles and microorganisms in the alveoli, whereas IM are believed to function in regulating tissue fibrosis, inflammation and antigen presentation (2). Marginated vascular macrophages appear to function in the crosstalk between antigen presenting cells 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/macrophage 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, alveolar macrophages 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).

Macrophages in the tissues of rodents have been well characterized, but the genetic (10), physiological, anatomical (11), immunological (12), and behavioral (13) differences between rodents and humans complicate translation of results from mice to humans, especially in relation to studies about AIDS, 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 ante-mortem 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 BAL, but macrophage subsets obtained from BAL are primarily AM and rarely contain IM (17). The purpose of this study, therefore, was to characterize IM 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 years old were used for these studies. All animal procedures were performed according to the "NIH Guide for the Care and Use of Laboratory Animals" and were approved by the Tulane University Institution Animal Care and Use Committee. The 5-bromo-2-deoxyuridine (BrdU; Sigma Aldrich, St. Louis, MO) was prepared at 30 mg/ml in phosphate-buffered saline (PBS, pH 7.2, Ca/Mg-free; Mediatech, Manassas, VA) and filter sterilized. In some studies, another thymidine analogue, 5-ethynyl-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 hr before and several time points after BrdU injection (i.e. 24 hr, 48 hr, and 7 days) for evaluation of monocyte turnover rates. BAL samples were obtained by bronchoscopy 48 hr and/or 7 days later as indicated. At necropsy, lung tissue (approximately 3 cm^3) was obtained 48 hr after BrdU injection.

Isolation of immune system cells from lung

Lung tissues were digested in medium containing 200 U/ml type IV collagenase (Worthington Biochemical, cat no. 4189; Lakewood, NJ) and 0.05 mg/ml DNAase I (Roche Applied Science, cat no.10104159001; Indianapolis, IN) to obtain single cell suspensions as described in the online data Supplemental Fig. S1A. Classically, BAL has been used to recover AM and these cells also could be recovered by lung wash (agitation) of tissues recovered during lung resection surgery(18) as diagrammed in Supplemental Fig. S1A. AM were obtained via BAL using bronchoscopy or by lung wash from 2 cm^3 sections of lung tissue from which visible bronchi were removed, followed by three rinses with 30 ml of RPMI1640 (Cellgro; Manassas, VA) containing 10% of fetal calf serum (Gibco cat no. 26140-079; Grand Island, NY, USA) and 100 IU/ml of penicillin/streptomycin (EMD Millipore; Billerica, MA). Washed lung tissues were diced further into approximately 0.5 mm-thick sections with sterile scalpels, washed at least five times with 30 ml of RPMI1640 containing 5% of fetal calf serum and 100 IU/ml of penicillin/streptomycin or until the filtrate solution appeared clear, to remove cells from the alveolar spaces and blood vessels (Supplemental Fig. S1A). To isolate interstitial cells (Supplemental Fig. S1A), the remaining lung tissue was further digested in 30 ml of RPMI1640 containing 5% fetal calf serum, 100 IU/ml penicillin/streptomycin, 2 mM L-glutamine (Cellgro; Manassas, VA), 25 mM HEPES (Molecular Biology; Carlsbad, CA), 200 U/ml type IV collagenase (Worthington Biochemical, cat no. 4189; Lakewood, NJ) and 0.05 mg/ml DNAase I (Roche Applied Science, cat no.10104159001; 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 (GE Healthcare, cat no. 17-0891-01; Boston, MA) at 2000 rpm for 20 min (Allegra X-12R; Beckman, CA, USA). IC 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 BAL or 50,000 sorted cells by centrifugation (Shandon Cytospin 3, Thermo Electron Corporation) at 400x *g* for 3 min, and slides were stained with Wright-Giemsa. Differential counting was performed under light microscopy at 200x magnification.

Flow cytometry and data analysis

Two hundred microliters of whole blood or 10^6 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 3-laser FACS Aria (Becton Dickinson; San Jose, USA)(19). EdU staining was performed using the Click-iT™ EdU Pacific Blue Flow Cytometry Assay kit (Invitrogen, Cat: C-10418; Carlsbad, CA) according to the manufacturer's protocol. Antibodies used in these analyses are shown in Supplemental Table IA. Isotype-matched antibodies were used to confirm the phenotype of each cell population. Data analyses were performed with FlowJo (Version 9.6, TreeStar) software.

Immunofluorescence antibody staining and confocal microscopy

Lung tissue sections of 7 μM thickness were treated for immunefluorescence antibody staining as previously described (19). Antibodies used for immunohistochemistry were anti-CD163 (1:20; Clone: 10D6; Leica Biosystems Newcastle Ltd, Cat: CD163-L-U; Newcastle, UK), anti-CD206 (also named anti-MRC1; 1:100; Clone: polyclonal; Sigma Aldrich, Cat: HPA004114; St. Louis, MO), anti-Caveolin-1 (1:100; Clone: polyclonal; Sigma Aldrich, Cat: C4490; St. Louis, MO) and anti-BrdU (1:50; Clone: BU1/75; Novus Biologicals, Cat: nb500-169; Littleton CO). Apoptotic cells were detected with the Click-iT® TUNEL Alexa Fluor® 647 Imaging Assay kit (Invitrogen, Cat. no. C10247; Carlsbad, CA) based on the manufacturer's protocol. DNase treated slides were used as positive controls and those treated with DNase but without EdUTP 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 emersion (40x, fluotar/NA 1.0) with 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 5×10^5 live cells per ml of RPMI1640 (Cellgro; Manassas, VA) containing 10% of fetal calf serum (Gibco cat no. 26140-079; Grand Island, NY, USA) and 100 IU/ml of penicillin/streptomycin (EMD Millipore; Billerica, MA) and plated at 1ml per well into a 12-well plate. After 2 hr incubation at 37°C and 5% CO_2, non-adherent 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 (Thermo Scientific, Cat# RIFNG100; Rockford, IL, USA), or medium with IFN-γ plus 100 ng/ml *Escherichia coli* serotype 0127:B8 LPS. After 4 hr stimulation, cells were harvested and stained with surface markers CD3-V500, CD8-V500, CD20-V500, CD163-PE, CD206-APC, CD11b-AL700, HLA-DR-PE-C7 and intracellular TNF-α-APC (Supplemental Table IA). Data analyses were performed with FlowJo (Version 9.6, TreeStar) software. The fold change in mean fluorescent intensity was compared to the medium-only treatment for expression of intracellular TNF-α among different treatments.

Statistical analyses

Student's *t*-test was used to compare the mean differences between groups using Graphpad Prism version 5.00 for Windows (GraphPad Software, San Diego.CA). $P < 0.05$ was considered statistically significant.

Results

AM 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 was 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, over 90% of cells from 11 healthy rhesus macaque BAL samples were observed to be AM (Fig 1A) which is consistent with results reported on lavage samples from healthy humans (21). Morphological analysis of cells retrieved from fluorescence-activated cell sorting (FACS) revealed that macaque AM exhibited high auto-fluorescence due to their large size, as also observed for humans (21). Rhesus AM expressed HLA-DR^{hi}, $CD11b^{int}$, scavenger receptor CD163 and mannose

receptor CD206 as indicated in P1 of Fig. 1B. Epithelial cells (EC), on the other hand, exhibited low auto-fluorescence as shown in P2 of Fig. 1B. Moreover, the level of CD45 expression was used to confirm and discriminate EC from macrophages/lymphocytes (data not shown). Small lymphocytes, red blood cells and cell debris were HLA-DR^{-} CD11b^{-}, and belonged to the population of cells indicated as P3 in Fig. 1B. Since AM (P1) seemed to comprise at least two sub-populations, we sorted by FACS into AM-H (CD206^{hi}CD163^{hi}) and AM-L (CD206^{int}CD163$^{int.}$) and further analyzed by Wright-Giemsa staining. No morphological differences were observed except that AM-H were larger (FSC) and more granular (SSC) than AM-L (Supplemental Fig. S2).

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

Previous reports indicated that at least two populations of macrophages (AM and IM) have been identified in lung tissue of humans and mice (4). However, BAL samples contained only AM (Fig 1A and 1B). To determine the proportion of lung AM and IM in rhesus macaques, cell populations isolated from sections of approximately 2 cm^3 from various anatomical sites of lung tissue in relation to areas indicated on a silicone rubber cast of a normal monkey lung (Supplemental Fig. S1B) 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. S1C). Therefore, we performed the remaining analyses from lung tissue obtained from area R1 (Supplemental Fig. S1B). 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^{+}), AM (HLA-DR^{hi}, CD11b^{int}, CD163^{+}, CD206^{+}) and IM (HLA-DR^{hi}, CD11b^{hi}, CD163^{+}); Two populations of DC also were identified that included mDC (HLA-DR^{int}, CD11b^{int}, CD11c^{+}) and pDC (HLA-DR^{int}, CD11b^{dim}, 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 more than 55% and 10% of these were AM and IM, respectively (Fig 2B).

Spatial distribution of AM and IM in lung tissue

To corroborate the flow cytometry assessment of lung tissue, immunohistochemistry and confocal imaging were performed to determine the spatial distribution of the AM and IM using antibody panels: Panel I included BrdU-FITC, CD163-PE, CD14-ECD, CD4-PCP-Cy5.5, HLA-DR-PE-C7, CD206-APC, CD11b-AL700, CD16-APC-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-C7, CD11c-APC, CD11b-AL700, CD206-APC-C7, 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 antibody, 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+ AM were located almost exclusively in the alveoli of the lung and were larger than IM (Fig 3A and D). CD163+ CD206−/dim IM were located in the interstitial spaces of the lung tissue (Fig 3A, B, and C). IM were more frequently observed in the peribronchovascular and subpleural regions of the lung (Fig 3B, C, E and F). 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 Figure 2B.

High turnover of IM but not AM during steady state homeostasis

To evaluate potential functional differences and developmental relationships among blood monocytes ($CD14^+$), IM and AM, expression of 28 cellular markers (Supplemental Table IB) was examined on immunological cells of whole lung tissue. The phenotype of IM 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 IM or tissue macrophages (Fig 4). Moreover, monocytes could be divided into $CD36^{hi}$ and $CD36^{low}$ subpopulations but AM and IM both expressed high levels of CD36 (Fig 4). These phenotype relationships suggest that blood monocytes could be the direct precursor of IM. In addition, AM were easily distinguished from IM 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 hours 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 hr or later. To better characterize the relationship between blood monocytes, IM, and AM, BrdU was injected i.v. and blood specimens were collected 24 hr and 48 hr later. Lung tissues were obtained at necropsy 48 hr after BrdU injection. Single cell suspensions were prepared for analysis of IM and AM. More than 35% of IM and monocytes stained BrdU+, but only 1.518% (± 0.24) of AM were stained BrdU+ 48 hr later (Fig 5A and B). These data suggest that IM originate from blood monocytes and exhibit a relatively short life-span during steady state homeostasis. To verify that IM differentiate from monocytes rather than self-renew within the lung, IM were isolated 24 hr after BrdU injection and were observed to stain negative for BrdU (data not shown). Moreover, AM in BAL samples obtained 1, 2, 7, 21 and 28 days after BrdU injection exhibited only marginal levels of staining throughout these time points(Supplemental Fig. S3), further corroborating that AM exhibit a slower turnover rate and appear to be longer-lived cells in the lung alveolar spaces during steady state homeostasis.

To determine if apoptosis serves as a feedback mechanism to regulate macrophage population size, terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) was applied to assess apoptosis of AM and IM in lung tissues of four animals. Results in Figure 5C and 5D demonstrated that more than 22.41% (± 4.010) of IM labeled with TUNEL that was significantly higher than the 5.50% (± 1.299) of AM that labeled with TUNEL ($P = 0.007$). This suggests that apoptosis represents a potential mechanism for regulating numbers of IM in the lungs of rhesus macaques during steady state.

Monocytes/IM are precursors to AM

Our data clearly indicated that in steady state AM 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 measure the kinetics of AM in the alveolar space. BrdU (or EdU) was injected followed by BAL two days post BrdU injection and again seven days later (i.e. 5 days after the first BAL) to determine the changes in the alveolar spaces. As shown in Fig. 6A, 18.12% (± 1.545) of AM recovered from BAL 5 days after the initial BAL removal of AM exhibited staining for BrdU. This was significantly higher than the 2.64% (± 0.422) of

labeled AM during steady state homeostasis on day 2 ($P < 0.0001$) as shown in Fig. 6B. These results suggested an increased influx of AM from 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 IM but not in AM

To examine a functional difference between AM and IM, *ex vivo* classical macrophage activation signals, IFN-γ plus LPS, were applied to single lung cell isolates from the lung of rhesus macaque followed by flow cytometry analysis of intracellular TNF-α expression (Fig 7). The $CD206^{+}$AM expressed relatively higher basal levels of TNF-α compared to $CD206^{-}$IM (Fig 7A) in the presence of medium only that increased minimally following *ex vivo* IFN-γ plus LPS treatment (1.552 ± 0.251fold, n=3; P=0.0886; Fig 7B). Conversely, the expression of TNF-α in IM increased significantly following *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 IM or AM.

Discussion

Characterizing the biology of all lung macrophage subsets in healthy rhesus macaques is important 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 AM are the predominant immune cells in the lung, IM 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 IM maintain a relatively higher turnover rate, are shorter-lived in steady state than are AM; and *ex vivo* IFN-γ plus LPS treatment significantly increased intracellular expression of TNF-α in IM but not in AM. In contrast, AM exhibited relatively lower turnover during steady state homeostasis than did IM or blood monocytes. In addition, AM were reported to be longer-lived cells in lungs of mice (26, 28) and humans (29).

IM in lung tissues of rhesus macaques resembled blood monocytes phenotypically and were CD11b+, HLA-DR+, Mac387+, CD163+, CD14+, and CD206−. In steady state, IM exhibited higher turnover rates that were slightly lower than that of blood monocytes, suggesting that monocytes are the direct precursor of the lung IM. Moreover, the IM population size appeared to be regulated by apoptosis as indicated by more TUNEL+ IM than AM.

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

These findings suggest that IM are derived from blood monocytes and can serve as intermediates for differentiation into AM in primates as previously described in mice (31). It is still possible that the broncho-alveolar 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 AM can proliferate locally for self-maintenance under steady homeostasis, we were not able to directly demonstrate the self-renewal of AM in macaques. We did not observe BrdU+ AM and IM at 24h post BrdU injection when BrdU+ monocytes were detected in the blood. The appearance of BrdU+ blood monocytes, however, always preceded the appearance of BrdU+ IM in this study, suggesting a chronological sequence of differentiation from monocytes to interstitial macrophages. Furthermore, we observed no increased incorporation of BrdU+ by AM after 48 hr, 7 days, 21 days or 28 days (Supplement Fig S3) post 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 EGF-like molecule containing a mucin-like receptor (EMR)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 AM of rhesus macaques may self-renew as occurs in mice, but the rate of AM turnover would be much slower due to the low rate of BrdU incorporation observed in this study.

Perhaps more importantly, the higher turnover of IM and negligible replacement of AM during steady state strongly argues that the continuous availability of IM in lung tissue is required to maintain and re-establish homeostasis. The significant increase in TNF-α production in IM, but not AM, in response to IFNγ plus LPS classical macrophage activation stimuli *ex vivo* provides additional support that IM and AM represent different macrophage subsets with different functional properties in the lung and emphasizes the importance in studying IM and AM independently in a physiologically-relevant model to better understand pathogenesis of pulmonary diseases such as interstitial lung diseases (ILD) 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 IM in addition to AM 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 macaque are expected to provide 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 AM and IM) 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 IM exhibited high turnover and apoptosis, and were rapidly replenished with blood monocytes in normal animals supporting a critical role of IM in protection against continuous pathogen exposure from the environment. This was also supported by our recent findings that massive SIV infection and destruction of IM in the SIV-infected macaques correlates with AIDS disease progression and pulmonary tissue damage (unpublished). 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 non-infectious conditions such as ILD, pulmonary hypertension and COPD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

AM	Alveolar macrophages
BAL	bronchoalveolar lavage
IM	interstitial macrophages
TUNEL	TdT-mediated dUTP nick end labeling
BrdU	5-bromo-2-deoxyuridine
EdU	5-ethynyl-2′-deoxyuridine
ILD	interstitial lung diseases

References

1. Silverstein AM. Ilya Metchnikoff, the phagocytic theory, and how things often work in science. Journal of leukocyte biology. 2011; 90:409–410. [PubMed: 21880643]
2. Schneberger D, Aharonson-Raz K, Singh B. Monocyte and macrophage heterogeneity and Toll-like receptors in the lung. Cell and tissue research. 2011; 343:97–106. [PubMed: 20824285]
3. St-Laurent J, Turmel V, Boulet LP, Bissonnette E. Alveolar macrophage subpopulations in bronchoalveolar lavage and induced sputum of asthmatic and control subjects. The Journal of asthma : official journal of the Association for the Care of Asthma. 2009; 46:1–8. [PubMed: 19191129]
4. Laskin DL, Weinberger B, Laskin JD. Functional heterogeneity in liver and lung macrophages. Journal of leukocyte biology. 2001; 70:163–170. [PubMed: 11493607]
5. Barletta KE, Cagnina RE, Wallace KL, Ramos SI, Mehrad B, Linden J. Leukocyte compartments in the mouse lung: distinguishing between marginated, interstitial, and alveolar cells in response to injury. Journal of immunological methods. 2012; 375:100–110. [PubMed: 21996427]
6. Fitting C, Dhawan S, Cavaillon JM. Compartmentalization of tolerance to endotoxin. The Journal of infectious diseases. 2004; 189:1295–1303. [PubMed: 15031800]
7. Gordon S, Hamann J, Lin HH, Stacey M. F4/80 and the related adhesion-GPCRs. European journal of immunology. 2011; 41:2472–2476. [PubMed: 21952799]
8. Hamann J, Koning N, Pouwels W, Ulfman LH, van Eijk M, Stacey M, Lin HH, Gordon S, Kwakkenbos MJ. EMR1, the human homolog of F4/80, is an eosinophil-specific receptor. European journal of immunology. 2007; 37:2797–2802. [PubMed: 17823986]
9. Han X, Li X, Yue SC, Anandaiah A, Hashem F, Reinach PS, Koziel H, Tachado SD. Epigenetic regulation of tumor necrosis factor alpha (TNFalpha) release in human macrophages by HIV-1 single-stranded RNA (ssRNA) is dependent on TLR8 signaling. The Journal of biological chemistry. 2012; 287:13778–13786. [PubMed: 22393042]
10. Church DM, Goodstadt L, Hillier LW, Zody MC, Goldstein S, She X, Bult CJ, Agarwala R, Cherry JL, DiCuccio M, Hlavina W, Kapustin Y, Meric P, Maglott D, Birtle Z, Marques AC, Graves T, Zhou S, Teague B, Potamousis K, Churas C, Place M, Herschleb J, Runnheim R,

Forrest D, Amos-Landgraf J, Schwartz DC, Cheng Z, Lindblad-Toh K, Eichler EE, Ponting CP. Lineage-specific biology revealed by a finished genome assembly of the mouse. PLoS biology. 2009; 7:e1000112. [PubMed: 19468303]

11. Yamanaka A, Kimura F, Takebayashi A, Kita N, Takahashi K, Murakami T. Primate model research for endometriosis. The Tohoku journal of experimental medicine. 2012; 226:95–99. [PubMed: 22245765]
12. Mestas J, Hughes CC. Of mice and not men: differences between mouse and human immunology. J Immunol. 2004; 172:2731–2738. [PubMed: 14978070]
13. Watson KK, Platt ML. Of mice and monkeys: using non-human primate models to bridge mouse- and human-based investigations of autism spectrum disorders. Journal of neurodevelopmental disorders. 2012; 4:21. [PubMed: 22958282]
14. Kaushal D, Mehra S, Didier PJ, Lackner AA. The non-human primate model of tuberculosis. Journal of medical primatology. 2012; 41:191–201. [PubMed: 22429048]
15. Messaoudi I, Estep R, Robinson B, Wong SW. Nonhuman primate models of human immunology. Antioxidants & redox signaling. 2011; 14:261–273. [PubMed: 20524846]
16. Yuan Q, Zhou Z, Lindell SG, Higley JD, Ferguson B, Thompson RC, Lopez JF, Suomi SJ, Baghal B, Baker M, Mash DC, Barr CS, Goldman D. The rhesus macaque is three times as diverse but more closely equivalent in damaging coding variation as compared to the human. BMC genetics. 2012; 13:52. [PubMed: 22747632]
17. Fathi M, Johansson A, Lundborg M, Orre L, Skold CM, Camner P. Functional and morphological differences between human alveolar and interstitial macrophages. Experimental and molecular pathology. 2001; 70:77–82. [PubMed: 11263950]
18. Hoppstadter J, Diesel B, Zarbock R, Breinig T, Monz D, Koch M, Meyerhans A, Gortner L, Lehr CM, Huwer H, Kiemer AK. Differential cell reaction upon Toll-like receptor 4 and 9 activation in human alveolar and lung interstitial macrophages. Respiratory research. 2010; 11:124. [PubMed: 20843333]
19. Hasegawa A, Liu H, Ling B, Borda JT, Alvarez X, Sugimoto C, Vinet-Oliphant H, Kim WK, Williams KC, Ribeiro RM, Lackner AA, Veazey RS, Kuroda MJ. The level of monocyte turnover predicts disease progression in the macaque model of AIDS. Blood. 2009; 114:2917–2925. [PubMed: 19383966]
20. Kastello MD, Emmert AD, Denson RF, Kishimoto RA. Recovery of alveolar macrophages from rhesus and cynomolgus macaques by lung lavage. American journal of veterinary research. 1979; 40:271–273. [PubMed: 111588]
21. Heron M, Grutters JC, ten Dam-Molenkamp KM, Hijdra D, van Heugten-Roeling A, Claessen AM, Ruven HJ, van den Bosch JM, van Velzen-Blad H. Bronchoalveolar lavage cell pattern from healthy human lung. Clinical and experimental immunology. 2012; 167:523–531. [PubMed: 22288596]
22. Lau SK, Chu PG, Weiss LM. CD163: a specific marker of macrophages in paraffin-embedded tissue samples. American journal of clinical pathology. 2004; 122:794–801. [PubMed: 15491976]
23. Van den Heuvel MM, Tensen CP, van As JH, Van den Berg TK, Fluitsma DM, Dijkstra CD, Dopp EA, Droste A, Van Gaalen FA, Sorg C, Hogger P, Beelen RH. Regulation of CD 163 on human macrophages: cross-linking of CD163 induces signaling and activation. Journal of leukocyte biology. 1999; 66:858–866. [PubMed: 10577520]
24. Staples KJ, Hinks TS, Ward JA, Gunn V, Smith C, Djukanovic R. Phenotypic characterization of lung macrophages in asthmatic patients: overexpression of CCL17. The Journal of allergy and clinical immunology. 2012; 130:1404–1412. e1407. [PubMed: 22981793]
25. Smetana K Jr, Mericka O, Saeland S, Homolka J, Brabec J, Gabius HJ. Diagnostic relevance of Langerin detection in cells from bronchoalveolar lavage of patients with pulmonary Langerhans cell histiocytosis, sarcoidosis and idiopathic pulmonary fibrosis. Virchows Archiv : an international journal of pathology. 2004; 444:171–174. [PubMed: 14722767]
26. Maus UA, Janzen S, Wall G, Srivastava M, Blackwell TS, Christman JW, Seeger W, Welte T, Lohmeyer J. Resident alveolar macrophages are replaced by recruited monocytes in response to endotoxin-induced lung inflammation. American journal of respiratory cell and molecular biology. 2006; 35:227–235. [PubMed: 16543608]

27. Taut K, Winter C, Briles DE, Paton JC, Christman JW, Maus R, Baumann R, Welte T, Maus UA. Macrophage Turnover Kinetics in the Lungs of Mice Infected with Streptococcus pneumoniae. American journal of respiratory cell and molecular biology. 2008; 38:105–113. [PubMed: 17690327]
28. Murphy J, Summer R, Wilson AA, Kotton DN, Fine A. The prolonged life-span of alveolar macrophages. American journal of respiratory cell and molecular biology. 2008; 38:380–385. [PubMed: 18192503]
29. Nakata K, Gotoh H, Watanabe J, Uetake T, Komuro I, Yuasa K, Watanabe S, Ieki R, Sakamaki H, Akiyama H, Kudoh S, Naitoh M, Satoh H, Shimada K. Augmented proliferation of human alveolar macrophages after allogeneic bone marrow transplantation. Blood. 1999; 93:667–673. [PubMed: 9885229]
30. Misharin AV, Scott Budinger GR, Perlman H. The lung macrophage: a Jack of all trades. American journal of respiratory and critical care medicine. 2011; 184:497–498. [PubMed: 21885631]
31. Landsman L, Jung S. Lung macrophages serve as obligatory intermediate between blood monocytes and alveolar macrophages. J Immunol. 2007; 179:3488–3494. [PubMed: 17785782]
32. Yona S, Kim KW, Wolf Y, Mildner A, Varol D, Breker M, Strauss-Ayali D, Viukov S, Guilliams M, Misharin A, Hume DA, Perlman H, Malissen B, Zelzer E, Jung S. Fate mapping reveals origins and dynamics of monocytes and tissue macrophages under homeostasis. Immunity. 2013; 38:79–91. [PubMed: 23273845]
33. Hashimoto D, Chow A, Noizat C, Teo P, Beasley MB, Leboeuf M, Becker CD, See P, Price J, Lucas D, Greter M, Mortha A, Boyer SW, Forsberg EC, Tanaka M, van Rooijen N, Garcia-Sastre A, Stanley ER, Ginhoux F, Frenette PS, Merad M. Tissue-resident macrophages self-maintain locally throughout adult life with minimal contribution from circulating monocytes. Immunity. 2013; 38:792–804. [PubMed: 23601688]
34. Seok J, Warren HS, Cuenca AG, Mindrinos MN, Baker HV, Xu W, Richards DR, McDonald-Smith GP, Gao H, Hennessy L, Finnerty CC, Lopez CM, Honari S, Moore EE, Minei JP, Cuschieri J, Bankey PE, Johnson JL, Sperry J, Nathens AB, Billiar TR, West MA, Jeschke MG, Klein MB, Gamelli RL, Gibran NS, Brownstein BH, Miller-Graziano C, Calvano SE, Mason PH, Cobb JP, Rahme LG, Lowry SF, Maier RV, Moldawer LL, Herndon DN, Davis RW, Xiao W, Tompkins RG. Inflammation, and L. S. C. R. P Host Response to Injury. Genomic responses in mouse models poorly mimic human inflammatory diseases. Proceedings of the National Academy of Sciences of the United States of America. 2013; 110:3507–3512. [PubMed: 23401516]
35. Meyer KC, Raghu G. Bronchoalveolar lavage for the evaluation of interstitial lung disease: is it clinically useful? The European respiratory journal : official journal of the European Society for Clinical Respiratory Physiology. 2011; 38:761–769.
36. Manganelli P, Bertorelli G, Fietta P, Casalini A, Mori PA, Nervetti A, Pesci A. Interstitial lung disease in connective tissue inflammation. Evaluation by means of bronchoalveolar lavage. Recenti progressi in medicina. 1988; 79:424–429. [PubMed: 3247512]
37. Nilsson K, Henriksson R, Hellstrom S, Tengblad A, Bjermer L. Hyaluronan reflects the pre-fibrotic inflammation in irradiated rat lung: concomitant analysis of parenchymal tissues and bronchoalveolar lavage. International journal of radiation biology. 1990; 58:519–530. [PubMed: 1975613]
38. Demands for rhesus monkeys in biomedical research: a workshop report. ILAR journal / National Research Council, Institute of Laboratory Animal Resources. 2003; 44:222–235.
39. Gardner MB, Luciw PA. Macaque models of human infectious disease. ILAR journal / National Research Council, Institute of Laboratory Animal Resources. 2008; 49:220–255.

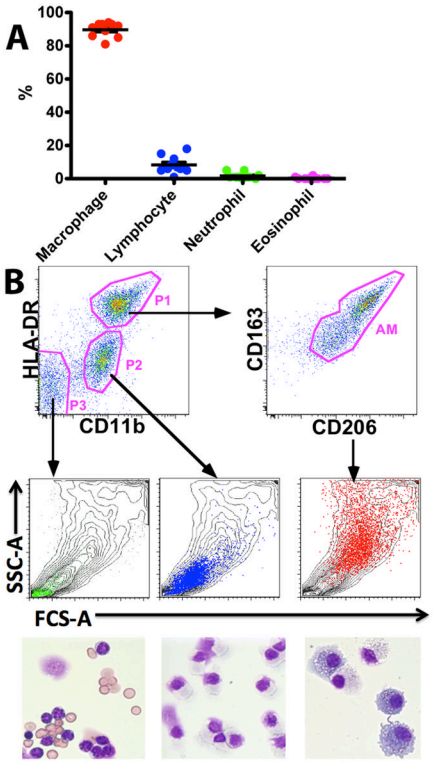

Fig 1. Identification of AM in BAL

Results in Panel A demonstrate the differential cell counts of BAL specimens from 11 healthy adult rhesus macaques. Cells recovered from BAL were stained, analyzed and sorted into three populations (P1, P2 and P3) by 3-laser FACSAria (Becton Dickinson) based on expression of HLA-DR and CD11b (myeloid cell markers) as shown in Panel B. Cytospins were prepared from each sorted fraction, stained with Wright-Giemsa, and imaged under 400X magnification. Results shown are representative of studies from three monkeys.

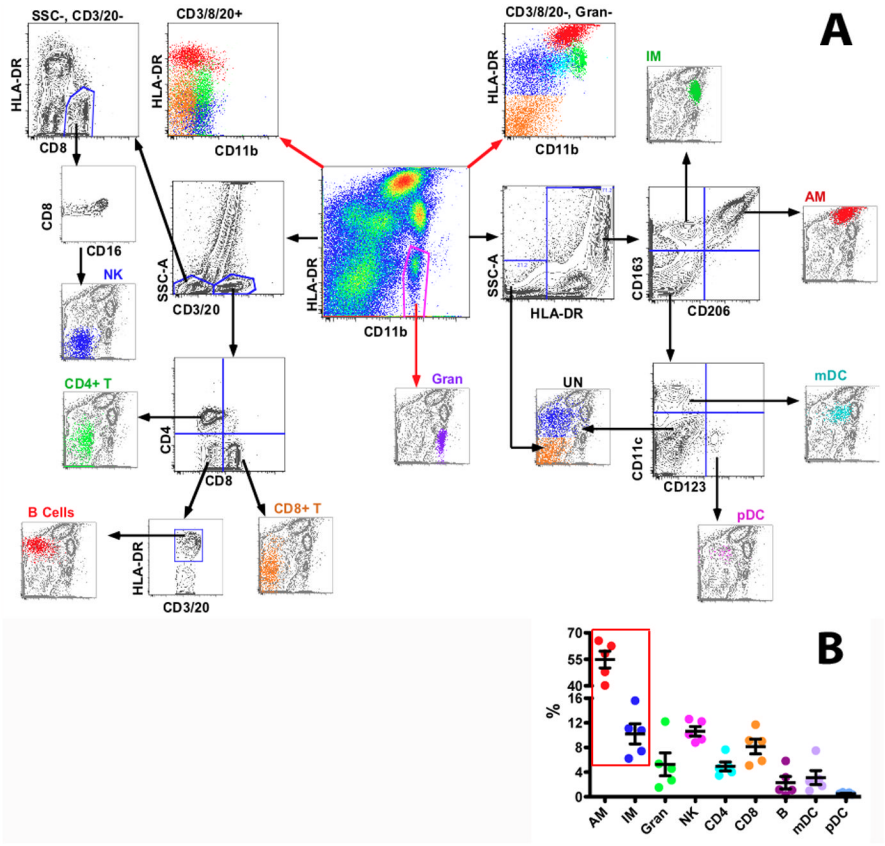

Fig 2. Macrophages are predominant cells of the immune system in healthy lung

Panel A shows the 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-DR^{hi}, CD11b^{+}, CD163^{+}, CD206^{+} cells were defined as AM. IM were identified as HLA-DR^{hi}, CD11b^{hi}, CD163^{+} cells. mDC were identified as CD11c+, HLA-DR+, CD163−, CD206− and pDC 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 (± st. dev.) of each cell population were determined from lung tissues of five rhesus macaques as shown in Panel B.

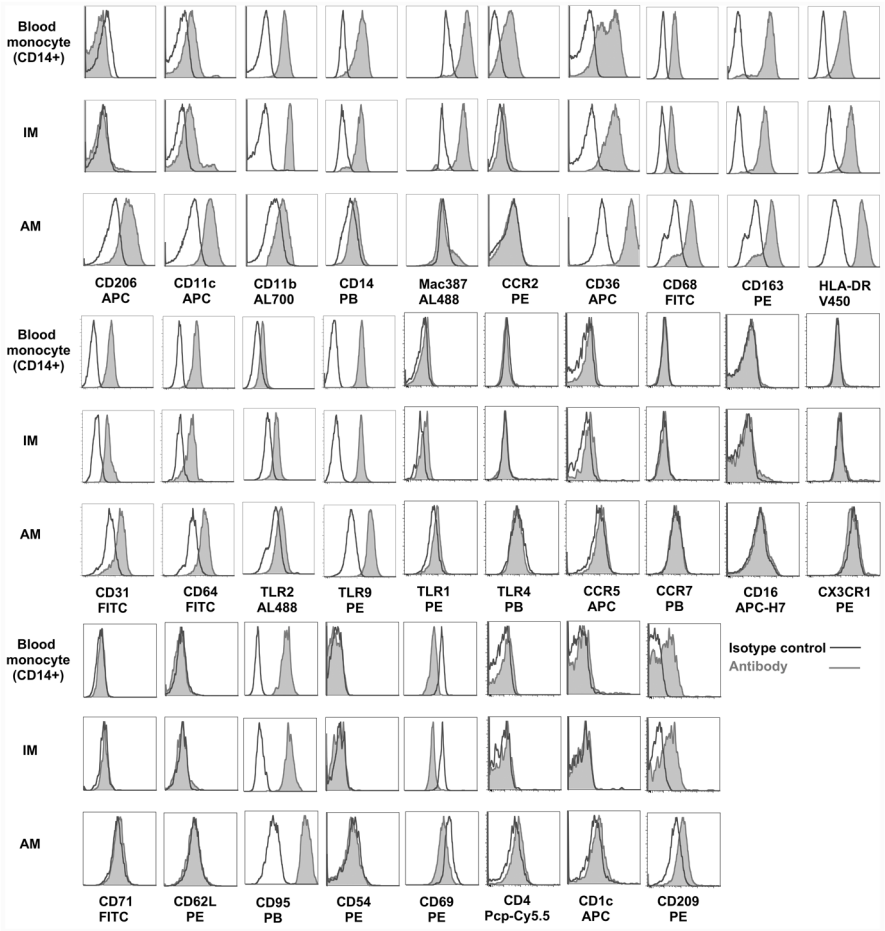

Fig 3. Confocal microscopy for the identification of AM and IM in healthy rhesus macaque lung tissue

Tissue sections from three different areas of the lung were analyzed: alveolar/interlobular (A&D), peribronchovascular (B&E) and subpleura (C&F). CD163 (macrophage marker, green), caveolin-1 (endothelium marker, red) and ToPro-3 (nucleic acid, blue) were used in Panels A, B, and C. CD206 (mannose receptor (AM marker), red), CD163 (green) and ToPro-3 (blue) were used in Panels D, E, and F. White arrows (→) indicate IM (CD163+ cells) outside the vessels. Stars (*) indicate AM (CD163+CD206+) that were located exclusively in the alveolar space. Sections shown are representative of three monkeys and were imaged using a Leica TCS SP2 confocal microscope equipped with three lasers (Leica Microsystems) at 400x final magnification under oil (40X objective, fluotar/NA 1.0).

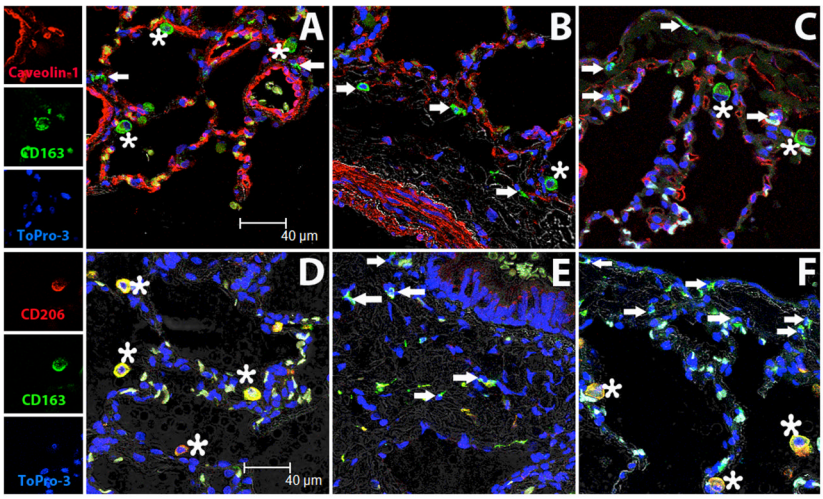

Fig. 4. Phenotype differences between AM, IM and monocytes

Blood monocytes, IM and AM were stained with antibodies (Supplemental Table IA) for flow cytometry analyses. Black lines represent isotype control antibody staining and the filled gray lines indicate specific antibody staining. The histograms are representative of at least three healthy rhesus macaques. The results demonstrate that monocytes, IM and AM could be discerned from each other, but monocyte and IM were relatively similar to each other.

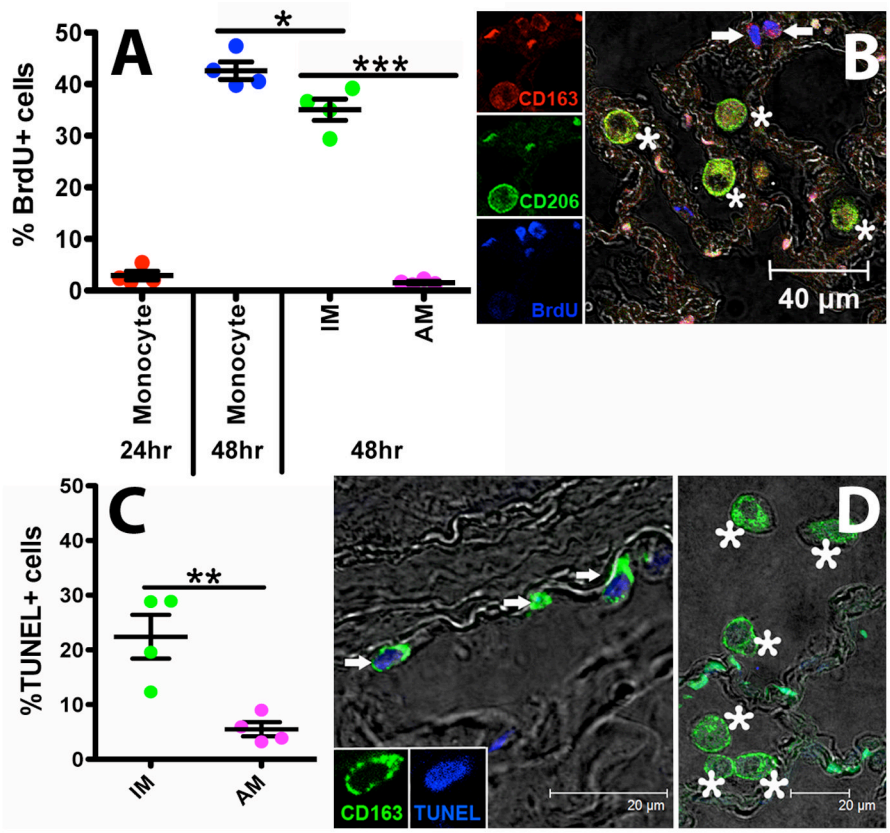

Fig 5. IM but not AM exhibit high turnover during steady state homeostasis

Cell turnover was reflected by measuring the uptake of BrdU by AM, IM or monocytes in relation to the total population. As shown in Panel A, BrdU staining was highest in monocytes and IM 48 hr after BrdU injection and was low in AM after 48 hr and in monoctyes after 24hr (n=4). Staining 24 hr 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 Panel 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). Stars (*) indicate CD163+CD206+ AM with no BrdU staining. Arrows (→) indicate CD163 single positive IM stained with BrdU. This experiment was performed using samples collected from four different uninfected monkeys necropsied two days after BrdU injection. Results in Panel C indicated that a significantly higher percent of IM than AM were undergoing apoptosis as measured by TUNEL staining. A confocal microscopy image in Panel D shows apoptotic (blue), CD163+ (green) macrophages in normal lung tissue and demonstrates high turnover of IM in relation to increased apoptosis. Arrows (→) indicate CD163 single positive IM stained with TUNEL. Stars (*) indicate AM (CD163+) with no TUNEL staining. Confocal images were acquired under an oil objective (63X, fluotar/NA 1.0) and are representative of studies from four monkeys (A&C).

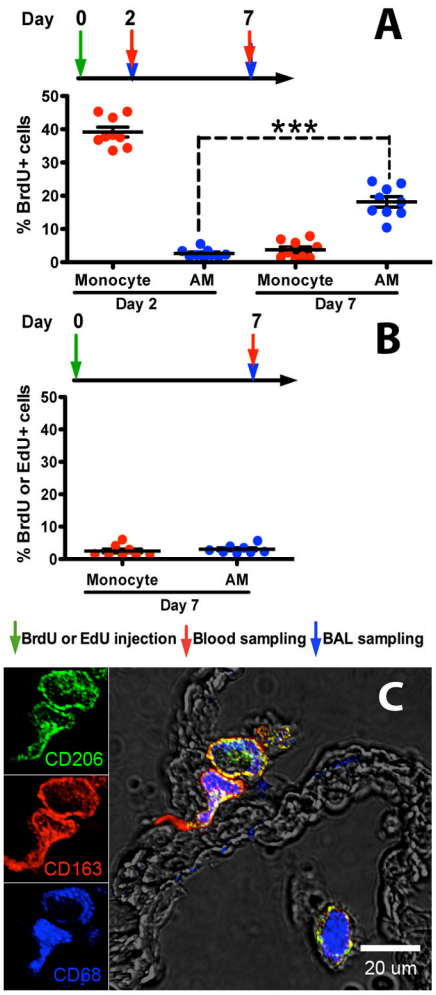

Fig 6. Monocytes/IM are precursors to AM

Rhesus macaques were injected with BrdU or EdU nucleic acid analogues and stained for uptake and macrophage markers as indicated. In the first experiment, BAL was performed on day 2 to remove AM and again on day 7 (i.e. five days later) to follow the turnover of the AM that were repopulating the alveolar space. Results in Panel A (n=8) demonstrated significant increases in repopulating AM turnover 5 days after mechanical removal of AM via BAL. If no initial BAL is performed on day 2, AM turnover was observed to remain low on day 7 as shown in Panel B (n=6). A macrophage undergoing transition from IM to AM is shown in Panel C and exhibits expression of macrophage markers CD68 (blue), CD206 (green) and CD163 (red). The image was captured under oil immersion (63X, fluotar/NA 1.0).

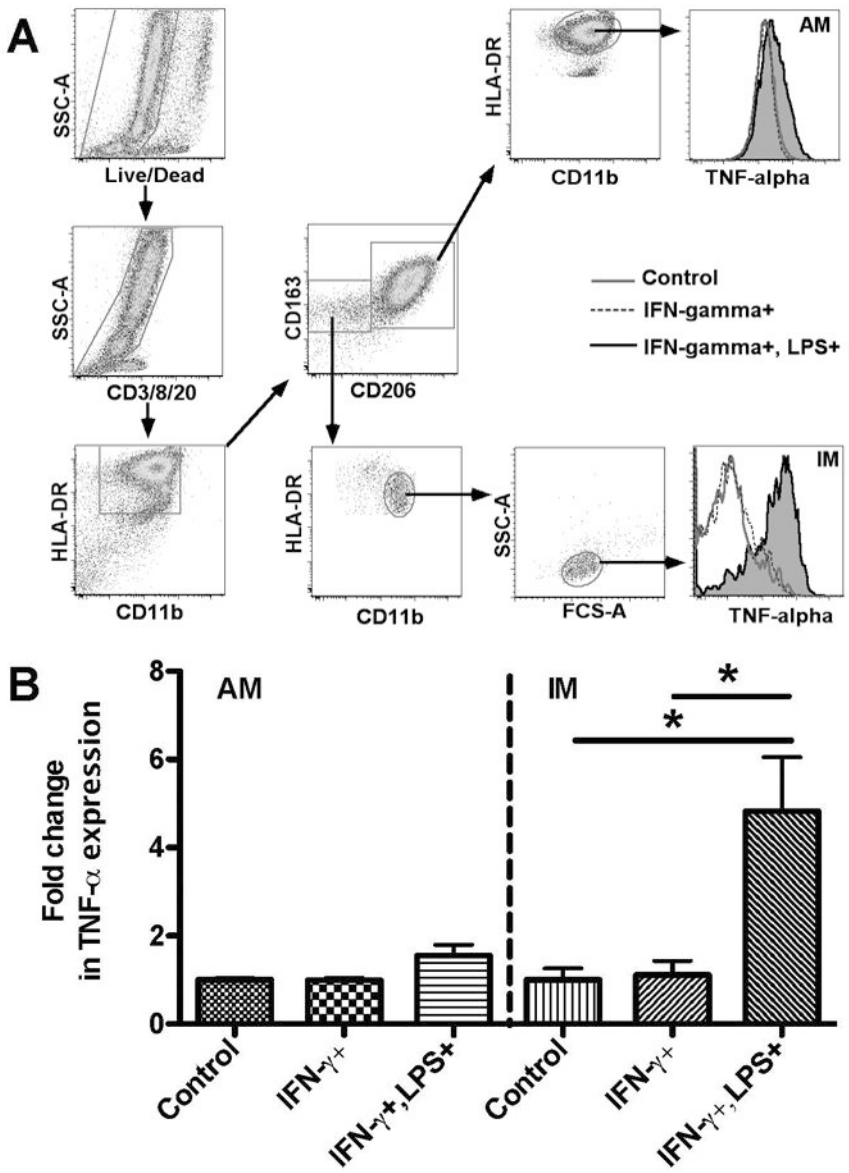

Fig 7. *Ex vivo* macrophage activation signaling with IFN-γ plus LPS significantly increased TNF-α expression in IM but not in AM

Adherent single cell isolates from the lung of uninfected rhesus macaque were stimulated with LPS and IFN-γ *ex vivo* for 4hr prior live/dead and antibody staining for flow cytometry analysis. Results in Panel A demonstrated the gating strategy for AM and IM, as well as representative histograms for the intracellular staining of TNF-α in AM and IM post stimulation from three uninfected monkeys. Results in Panel B show the mean fold-change (± st. dev.) in intracellular expression of TNF-α in AM and IM after LPS and IFN-γ treatment compared to untreated or IFN-γ alone controls (n=3).

Table 1

Ratio of AM:IM in normal lung tissue sections*

Animal ID	Alveolar		Interstitial		Peribroncho- vascular		Subpleural		Total AM	Total IM	AM: IM
	Large	Small	Large	Small	Large	Small	Large	Small			
GI53	511	14	**0**	44	**0**	88	**0**	39	525	171	3.07
EC61	1135	28	**0**	61	**0**	122	1	77	1163	261	4.45

*Observations were recorded from 20 fields per slide under 200x magnification at one slide per monkey.