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Distinct Macrophage Subpopulations Characterize Acute Infection and Chronic Inflammatory Lung Disease

Mubing Duan,*†‡ Waichu C. Li,* Ross Vlahos,§ Mhairi J. Maxwell,*,‡ Gary P. Anderson,§,* and Margaret L. Hibbs*‡

Although great progress has been made in delineating lung dendritic cell and lymphocyte subpopulations, similar advances in lung macrophages (MΦs) have been hampered by their intrinsic autofluorescence, cell plasticity, and the complexities of monocyte–MΦ compartmentalization. Using spectral scanning, we define alveolar MΦ autofluorescence characteristics, which has allowed us to develop an alternative flow cytometry method. Using this methodology, we show that mouse lung MΦs form distinct subpopulations during acute inflammation after challenge with LPS or influenza virus, and in chronic inflammatory lung disease consequent to SHIP-1 deletion. These subpopulations are distinguished by differential Mac-1 and CD11c integrin expression rather than classical M1 or M2 markers, and display differential gene signatures ex vivo. Whereas the resolution of acute inflammation is characterized by restoration to a homogenous population of CD11c<sup>high</sup>Mac-1<sup>neg/low</sup> MΦs reflective of lung homeostasis, chronic inflammatory lung disease associated with SHIP-1 deficiency is accompanied by an additional subpopulation of CD11c<sup>high</sup>Mac-1<sup>pos</sup> MΦs that tracks with lung disease in susceptible genetic background SHIP-1<sup>-/-</sup> animals and disease induction in chimeric mice. These findings may help better understand the roles of MΦ subpopulations in lung homeostasis and disease.

MΦs maintain organ homeostasis, facilitate host defense and wound healing, but also underlie the pathogenesis of many chronic diseases (1). This functional dichotomy arises from their intrinsic plasticity, causing polarization into distinct functional phenotypes depending on the environmental stimuli. For instance, classically activated or M1-like MΦs become more proinflammatory and bactericidal in the presence of IFN-γ and LPS, whereas alternatively activated or M2-like MΦs are immunoregulatory, and can promote wound healing and modify the extracellular matrix through the secretion of proteases and growth factors (2, 3). Because there is complex overlap between tissue homeostasis, inflammation, and disease pathogenesis, it is now thought that distinct MΦ subpopulations may exist that subserve their protective or pathogenic roles (1). A prime example may be in lungs, where residential alveolar MΦs (AMΦs) are simultaneously implicated to play many homoeostatic (4), proinflammatory (5, 6), and pathogenic roles (7, 8).

In healthy adult lungs, AMΦs exist as the predominant cell type (98%*) in the alveolar airspaces. There, they sit as a uniform, quiescent, and immunosuppressive population capable of pathogen phagocytosis and T cell recruitment in the early event of a bacterial or viral infection (9, 10). Cell turnover rate is slow (11) and is maintained by constitutively immigrating CCR2<sup>+</sup>Gr-1<sup>+</sup> resident monocytes (12, 13). In contrast, CCR2<sup>+</sup>Gr-1<sup>-</sup> inflammatory monocytes rapidly migrate into alveolar airspaces after lung infection and are believed to be the main effectors of acute lung injury and infection-related mortality (6, 14, 15). Increased AMΦs, however, are correlated with the severity of chronic obstructive pulmonary disease (COPD) (16), a debilitating chronic condition characterized by progressive irreversible airflow limitation and lung parenchyma destruction. Altered AMΦ behavior is also observed in animal models of COPD, chronic lung inflammation, and tumorigenesis (7, 8, 17, 18), making the behavior of lung MΦs an important field of study.

Unfortunately, efforts to identify AMΦ subsets in the lung microenvironment have been severely hampered by their intense cell autofluorescence (19), unlike inflammatory lung monocytes (or “exudate” lung MΦs), which are less autofluorescent, easier to study by flow cytometry, and thus better characterized. Altogether, this has created a gap in our understanding of AMΦ heterogeneity and their roles in the orchestration of host defense, inflammation, and disease. In this article, we demonstrate that AMΦ autofluorescence emission diminishes above 640 nm, enabling us to establish a modified flow cytometry method using fluorophore-conjugated Abs that emit at wavelengths (λs) >660 nm. Screening LPS-treated and influenza-infected C57BL/6 mice, we found that MΦ heterogeneity to be a prominent component of the acute lung immune response, distinctly characterized by three MΦ subpopulations present in the alveolar airspaces (CD11c<sup>high</sup>Mac-1<sup>neg/low</sup>...
“residential,” CD11c<sup>high</sup>Mac-1<sup>pos</sup> “intermediate,” and CD11c<sup>low</sup> Mac-1<sup>high</sup> monocytes). Subpopulations displayed distinct gene profiles independent of an exclusive M1 or M2 polarization signature, with restoration to the CD11c<sup>high</sup>Mac-1<sup>neg/low</sup> subpopulation marking resolution and the reinstatement of lung homeostasis. Chronic inflammatory lung disease was then investigated using SHIP-1–deficient mice, where AMΦ polarization was first identified as an effector of chronic lung disease and tumorigenesis (8). AMΦ subpopulation deregulation (marked by an additional subpopulation of residual CD11c<sup>high</sup>Mac-1<sup>pos</sup> AMΦs) was found to be a prominent novel feature of SHIP-1–deficient mice and tracked specifically with lung disease pathogenesis. Altogether, our studies suggest a novel role for AMΦ subpopulations and Mac-1 signaling in the regulation of AMΦ activation, acute infection, and chronic inflammatory lung disease.

Materials and Methods

**Animals**

SHIP-1<sup>−/−</sup> mice (20) were crossed onto the C57BL/6 or BALB/c background for seven generations (21). C57BL/6 or BALB/c mice were bred under specific pathogen-free conditions by the Ludwig Institute for Cancer Research (LICR, Melbourne, VIC, Australia). BL6.SJL-Ptp<sup>−/−</sup> mice were purchased from ARC (Perth, WA, Australia). Animal experiments were approved by the Animal Ethics Committees of the LICR/Department of Surgery and the University of Melbourne in line with National Health and Medical Research Council guidelines.

**LPS and Mem71 virus challenge**

Ten- to 12-wk-old mice were challenged with a 30 μl transnasal administration of E. coli LPS (10 μg/mouse, serotype O114:B4; Sigma) or Mem71 influenza virus, 2000 PFU/mouse, sublethal dose, and euthanized at 24 h, 72 h, and day 10. Bronchoalveolar lavage (BAL) cells were collected as described later. Control mice received 30 μl DMEM.

**Acute cigarette smoke exposure**

C57BL/6 mice were exposed to cigarette smoke for 4 consecutive days (22), euthanized on day 5, and BAL cells collected. Control mice were housed in control conditions (22). BAL, and thus gate out BAL debris. Anti-mouse Ly6g–PE (BD Biosciences) was used to negatively gate out all neutrophils, and all lymphocytes were gated out based on forward scatter (FSC) and side scatter (SSC). This allowed FACs analysis on the remaining F<sub>M</sub><sup>−</sup>/F<sub>M</sub><sup>+</sup> monocytes using anti-mouse CD11c-allophycocyanin, CD11c-biotin, Class II IA–β–biotin, CD36-allophycocyanin, CD124-biotin, and CD40–biotin (all from BD Biosciences); Mac-1–Per-Cy5.5, and CD23–Per-Cy7 (eBioscience); and F4/80–biotin and AF598–biotin (gift from J. Hamilton, University of Melbourne). Data were acquired on a FACSAria (BD Biosciences), which collected ≥5000 AMΦ events. Analysis was performed using FlowJo software (Mac V8.7.3). Total BAL cell counts were performed using a nucleocounter (Chemotec), with absolute cell numbers calculated as total BAL cell number × percentage of cell subpopulation as determined by FACs (using CD45<sup>−</sup>Ly6<sup>−</sup>Gr1<sup>−</sup> and FSC versus SSC profile for macrophages, and CD45<sup>−</sup>Ly6g<sup>+</sup> and FSC versus SSC profile for AMΦs).

Sorting of BAL AMΦs was performed on individual mouse samples using the staining approach described earlier. A total of ≥250,000 cells/subpopulation were sorted for cytokin analysis and quantitative RT-PCR (qRT-PCR).

**RNA isolation and qRT-PCR**

Sorted BAL cells were pelleted, resuspended in 350 μl RLT plus buffer + 2-ME (Qiagen), snap frozen in liquid nitrogen, and stored at −70°C. RNA was isolated using the RNeasy Plus Micro Kit (Qiagen), and quality and quantity were measured using NanoDrop. For each experiment, a consistent amount of RNA/sample (200–300 ng) was converted to cDNA (Superscript VILO cDNA Synthesis Kit; Invitrogen) and diluted 1:1 in RNase-free H<sub>2</sub>O. Genes of interest were screened using preoptimized and validated TaqMan assays. Reaction mix consisted of 2 μl cDNA, 0.5 μl target primer, and 5 μl TaqMan Universal PCR Mastermix diluted with 2.5 μl RNase-free H<sub>2</sub>O and run for 40 cycles under fast single-plex conditions (ABI 7900ht). Each reaction (18S RNA [Applied Biosystems]) was used as endogenous control for all samples. For each gene target, a no-RT negative control was used to check against nonspecific binding to genomic DNA. Cycle threshold (C<sub>T</sub>) values were calculated using automatic threshold detection. The relative expression of each target gene was calculated using the 2<sup>−ΔΔCT</sup> method (normalized to 18S control and relative to DMEM R control, or LPS R when DMEM R transcript undetectable). Results were calculated from a total of four independent experiments. All RNA samples were prepared together and run on a single plate to ensure that each independent experiment produced data all directly compared with control R.

**Statistical analysis**

Statistical analyses were performed using GraphPad Prism V.4.03. Values are expressed as mean ± SEM from two or more independent experiments. The unpaired two-tailed Student t test was used for all excepting qRT-PCR data, which were analyzed using the two-tailed Mann–Whitney U test.

**Results**

Spectral scanning directs new AMΦ characterization approach using flow cytometry

Although surface markers exist for the identification of lung dendritic cell and lymphocyte subpopulations, a standard and consistent approach to the study of AMΦs has yet to be identified due first to

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their intrinsically high autofluorescence. We used confocal microscopy to perform spectral scanning of AMΦ emission λs. AMΦs were significantly more autofluorescent than peritoneal MΦs, with emission peaking around the emission λs of traditional fluorophores FITC and PE, but not allophycocyanin (Fig. 1A, 1B). As expected, flow cytometry using traditional FITC- or PE-conjugated Abs produced only a small shift in positive (i.e., CD11c) staining because of the high background noise from AMΦ autofluorescence (Fig. 1C). Because this autofluorescence greatly diminishes at λ > 660 nm, fluorophores that emitted beyond this λ (i.e., allophycocyanin, Pe-Cy7, and allophycocyanin-Alexa Fluor 750) were then put under trial. These produced a clear separation between negative and positive AMΦ staining, and revealed that AMΦs were CD11c<sup>high</sup>Mac-1<sup>neg/low</sup> (Fig. 1C), assenting with previous studies of rat AMΦs where differential surface Ag staining could be discretely distinguished using allophycocyanin (23). Although clinical study has suggested that basal AMΦ autofluorescence derives from acquired tobacco tar exposure (24), we show that resting mouse AMΦs are intrinsically more autofluorescent compared with peritoneal MΦs, further emphasizing the necessity of our methodological approach.

We first tested a panel of M1 and M2 surface markers, but did not identify distinct AMΦ subpopulations (Supplemental Fig. 1A). Leukocyte integrins play an important role in monocyte migration and activation, and are differentially expressed by blood monocyte subsets (25). Thus, we assayed the expression of leukocyte integrins CD11a, CD11b (Mac-1), and CD11c in AMΦs from control and LPS-treated mice. Only Mac-1 expression was found to be differentially distributed in LPS-treated, but not control AMΦs (Fig. 1D). Using CD11c and Mac-1 as subpopulation markers, we then investigated the behavior of AMΦs in different respiratory conditions.

**AMΦ subpopulations identified after LPS and virus challenge in mice**

To detect AMΦ populations during acute lung infection, we first studied C57BL/6 mice 72 h post-LPS and Mem71 influenza virus challenge, because this is when initial lung neutrophilia subsides and MΦs become prominent. As expected, LPS challenge elicited an acute inflammatory response with alveolar airspace infiltration by mononuclear cells, especially near regions of pulmonary blood vessels (Fig. 2A). BAL MΦs, neutrophil, and lymphocyte numbers were significantly higher compared with DMEM-treated (i.e., control) mice (Fig. 2B), as reflected by the altered BAL cell FSC versus SSC (Fig. 2A).

We identified three BAL MΦ subpopulations in mice challenged with LPS or Mem71 influenza virus. In control mice, residual AMΦs were >95% CD11c<sup>high</sup>Mac-1<sup>neg/low</sup> (subpopulation R), and were c-fms<sup>pos</sup> and Ly6c<sup>neg</sup> (Fig. 2C). After LPS challenge, three MΦ subpopulations appeared in BAL: “residential” CD11c<sup>high</sup>Mac-1<sup>neg/low</sup> AMΦs (subpopulation R), CD11c<sup>high</sup>Mac-1<sup>pos</sup> AMΦs of an “intermediate” phenotype (subpopulation I), and a subpopulation of lung monocytes that were CD11c<sup>low</sup>Mac-1<sup>high</sup> (subpopulation Me). All subpopulations were c-fms<sup>pos</sup>, with only the monocyte subpopulation also Ly6c<sup>pos</sup> (Fig. 2C).

A differential MΦ signature was observed postinfection with Mem71 (H3N1) influenza virus. A smaller proportion of residential CD11c<sup>high</sup>Mac-1<sup>neg/low</sup> AMΦs (subpopulation R) was observed, CD11c<sup>high</sup>Mac-1<sup>pos</sup> intermediate MΦs (subpopulation I) were c-fms<sup>low</sup> but Ly6c<sup>pos</sup>, and there was a predominance of CD11c<sup>neg/low</sup>
FIGURE 2. Three lung MΦ subpopulations exist in acute lung inflammation. (A) MT-stained lungs of C57BL/6 mice 72 h post DMEM- (control), LPS-, and Mem71 virus-challenge (top panels). Red arrows indicate inflammatory cells in alveolar airspaces; black arrow indicates cellular infiltration from pulmonary vessels. Scale bar, 50 μm. BAL cell size (FSC) and granularity (SSC) from treated mice shown using flow cytometry (bottom panels). (B) Cellular composition of BAL from control, LPS-, and Mem71-infected mice 72 h postchallenge. Cell differentials determined by flow cytometry. *p < 0.05, **p < 0.005, ***p < 0.0001; n = 6–7 from three independent experiments. (C) Flow cytometry of Mac-1 versus CD11c of BAL MΦs in LPS-treated and Mem71-infected mice. Each subpopulation is gated and further assessed for c-fms and Ly6c expression; n = 6–7 from three independent experiments. (D) Cytospins of sorted lung MΦ subpopulations from control, LPS-treated, and Mem71-infected mice. Representative of three independent sorts. Scale bar, 20 μm. (E) qRT-PCR of sorted lung MΦ subpopulations for Mac-1, iNOS, Arg-1, MMP-12, IL-6, and IL-10 gene expression. All fold changes relative to control R (residential MΦ subpopulation from control mice), except for IL-10, which is calculated relative to LPS R because no IL-10 transcript was detectable in control R. Results are mean ± SEM, *p < 0.05, **p < 0.01; n = 4 from four independent experiments. Asterisk directly above a bar indicates p value compared with DMEM R. I, Intermediate; Me, monocyte-like postendotoxin; Mv, monocyte-like postvirus; R, residential.
Mac-1<sup>high</sup> monocytes (subpopulation Mv). Unlike LPS challenge, this monocyte subpopulation was Ly6c<sup>high</sup> but c-fms<sup>low</sup> (Fig. 2C).

MΦs are believed to polarize into either a classically activated M1-like or alternatively activated M2-like phenotype (2, 3), although how this manifests in the dynamic tissue microenvironment remains unclear. To assess this, we used our gating strategy to sort each AMΦ subpopulation and examined for differences in morphology and M1/M2 gene expression. Mac-1<sup>high</sup> (Me and Mv) monocytes were morphologically distinct from both residential and intermediate BAL MΦs, appearing as highly vacuolated cells with irregular-shaped nuclei (Fig. 2D, Supplemental Fig. 1B). For gene profiling, we focused on all AMΦ subpopulations from LPS-treated mice because LPS is a classical and well-studied M1 polarizing stimulus (3), together with subpopulation Mv in Mem71-infected mice because of its differential surface phenotype. Compared with residential AMΦs (subpopulation R) in control mice, Mac-1 gene expression was significantly upregulated in intermediate BAL MΦs and monocytes (subpopulation I and Me and Mv) as expected (Fig. 2E). Interestingly, both M1 and M2 signatures were upregulated in BAL monocytes from LPS- and Mem71-challenged mice. Inducible NO synthase (iNOS) was higher in all subpopulations compared with residential AMΦs from control mice. Uprogulation of the classical M2 marker arginase I (Arg I) was simultaneously observed in the intermediate and monocyte subpopulation from LPS-treated mice and in Mem71-infected monocytes (Fig. 2E). Both IL-6 (a known M1 MΦ cytokine) and IL-10 (the classical M2 MΦ cytokine) were simultaneously upregulated in LPS and Mem71 monocyte subpopulations compared with residential control. IL-6, but not IL-10, was also slightly but significantly upregulated in the intermediate AMΦ subpopulation of LPS-treated mice (Fig. 2E). Interestingly, matrix metalloproteinase 12 (MMP-12) was upregulated in all Mac-1<sup>pos</sup> BAL MΦs and monocytes (subpopulation I and Me and Mv) in challenged chimeric mice (Fig. 3A). Interestingly, both M1 and M2 signatures were upregulated in BAL monocytes from LPS- and Mem71-infected mice because of its differential surface phenotype. Compared with residential AMΦs (subpopulation R) in control mice, Mac-1 gene expression was significantly upregulated in intermediate BAL MΦs and monocytes (subpopulation I and Me and Mv) as expected (Fig. 2E). Interestingly, both M1 and M2 signatures were upregulated in BAL monocytes from LPS- and Mem71-challenged mice. Inducible NO synthase (iNOS) was higher in all subpopulations compared with residential AMΦs from control mice. Uprogulation of the classical M2 marker arginase I (Arg I) was simultaneously observed in the intermediate and monocyte subpopulation from LPS-treated mice and in Mem71-infected monocytes (Fig. 2E). Both IL-6 (a known M1 MΦ cytokine) and IL-10 (the classical M2 MΦ cytokine) were simultaneously upregulated in LPS and Mem71 monocyte subpopulations compared with residential control. IL-6, but not IL-10, was also slightly but significantly upregulated in the intermediate AMΦ subpopulation of LPS-treated mice (Fig. 2E). Interestingly, matrix metalloproteinase 12 (MMP-12) was upregulated in all Mac-1<sup>pos</sup> BAL MΦ subpopulations present after LPS treatment (Fig. 2E).

**Determination of AMΦ subpopulation origins**

We postulated that the CD11c<sup>high</sup>Mac-1<sup>pos</sup> MΦs (subpopulation I) observed during acute inflammation could represent residential AMΦs undergoing a continuum of Mac-1 upregulation. To confirm the origin of each MΦ subpopulation, we generated BM chimeras using CD45.1 donors and CD45.2 recipients, which we then challenged with LPS, Mem71, or DMEM (control). Background lung toxicity was observed, as CD45.2 chimeric mice had greater BAL debris and increased BAL MΦ heterogeneity compared with C57BL/6 mice (Supplemental Fig. 1A). Four weeks postirradiation and transfer of CD45.1 BM, 70–90% of BAL MΦs were CD45.2<sup>+</sup> in control chimeric mice, reflecting low residential AMΦ turnover (Fig. 3A, Supplemental Fig. 1C) as previously reported (11). In nonirradiated C57BL/6 (i.e., CD45.2 allotype mice), all peripheral blood leukocytes were CD45.2<sup>+</sup> (Supplemental Fig. 1C). In contrast, ≥95% of circulating blood monocytes were CD45.1<sup>+</sup> 4 wk postirradiation, demonstrating successful blood leukocyte reconstitution with CD45.1 donor BM (Supplemental Fig. 1C).

In control, LPS-, and Mem71-challenged CD45.2 chimeric mice, 70–90% of all CD11c<sup>high</sup>Mac-1<sup>pos</sup> residential AMΦs (subpopulation R) were CD45.2<sup>+</sup> as expected (Fig. 3A). BAL monocytes (subpopulation Me and Mv) in challenged chimeras were ≥95% CD45.1<sup>+</sup>, confirming their origin as newly recruited blood monocytes (Fig. 3A). Interestingly, in challenged chimeric mice, CD11c<sup>high</sup>Mac-1<sup>pos</sup> intermediate BAL MΦs (subpopulation I) contained equal proportions of CD45.1<sup>+</sup> and CD45.2<sup>+</sup> cells, indicating that ∼50% of the subpopulation had originated from residential AMΦs (Fig. 3A).

To determine whether residential AMΦ Mac-1 upregulation preceded monocyte recruitment during acute inflammation, we characterized BAL MΦ kinetics 24 h, 72 h, and 10 d post-LPS and Mem71 influenza treatment. Twenty-four hours post-LPS, only CD11c<sup>high</sup>Mac-1<sup>pos</sup> intermediates, but not CD11c<sup>low</sup>Mac-1<sup>high</sup>
FIGURE 4. Lung MΦ subpopulations exist in SHIP-1−/− mice with spontaneous lung disease. (A) MT-stained lungs of C57BL/6, acute cigarette smoke-exposed, and C57BL/6 SHIP-1−/− mice (top panels). Red arrow indicates myeloid infiltrates in SHIP-1−/− alveolar airspaces; black arrow indicates regions of collagen deposition. Scale bar, 50 µm. FSC versus SSC of BAL from respective mice (bottom panel). (B) BAL cell differentials as determined by flow cytometry. *p < 0.05, **p < 0.005, ***p < 0.0001; n = 7 from two independent experiments. (C) Mac-1 versus CD11c expression of BAL MΦs. Each MΦ subpopulation is further analyzed for c-fms and Ly6c expression; n = 6 from two independent experiments. (D) Transmitted light image (top panels) and autofluorescence emission (middle panels) of BAL MΦs. Respective BAL MΦ emission spectra (bottom panel). Results are mean ± SEM; *p < 0.005, **p < 0.0005, ***p < 0.0001; representative of two independent experiments, n = 4. Scale bar, 10 µm. (E) Cytospins of sorted BAL MΦ subpopulations. Representative of three independent sorts. Scale bar, 20 µm. (F) qRT-PCR of sorted C57BL/6 and SHIP-1−/− BAL MΦ subpopulations for Mac-1, iNOS, Arg-1, MMP-12, IL-6, and IL-10 gene expression. Fold changes relative to DMEM R of Fig. 2E, except for IL-10, which is Figure legend continues
monocytes, were additionally observed in BAL, suggesting that residential AMΦ Mac-1 upregulation occurs before monocyte recruitment during acute lung inflammation (Fig. 3B). By day 10, resolution of all lung MΦs to a single CD11c<sup>highMac-1<sup>neg/low</sup> residential AMΦ phenotype had occurred in LPS-treated mice. This contrasted greatly with influenza infection, where four discrete MΦ-like subpopulations still existed, further highlighting differences in MΦ subpopulation regulation during lung disease (Fig. 3B).

Identification of AMΦ subpopulations in chronic lung disease

SHIP-1 is a hematopoietic-specific 5′inositol phosphatase that represses the antiapoptotic and proinflammatory PI3K pathway (20, 26), which is of particular importance in MΦs. M2 AMΦ polarization was first identified in SHIP-1<sup>−/−</sup> mice that spontaneously developed lung disease (8), making SHIP-1<sup>−/−</sup> mice an invaluable model for examining MΦ-mediated mechanisms underlying chronic lung disease. To examine whether AMΦ subpopulations were a hallmark of SHIP-1<sup>−/−</sup> lung disease, we studied C57BL/6 SHIP-1<sup>−/−</sup> mice that spontaneously developed severe lung inflammation marked by the infiltration of large myeloid cells (Fig. 4A). Increased collagen deposition and alveolar wall thickening was frequently observed in SHIP-1<sup>−/−</sup> lung, especially surrounding regions of MΦ consolidation (Fig. 4A). SHIP-1<sup>−/−</sup> BAL cells were greatly heterogeneous as marked by their FSC versus SSC (Fig. 4A), with MΦ, neutrophil, and lymphocyte numbers significantly greater compared with age-matched C57BL/6 controls (Fig. 4B).

Instead of global M2 MΦ polarization, we observed AMΦ subpopulation deregulation in C57BL/6 SHIP-1<sup>−/−</sup> mice (Fig. 4C), which had residential-like CD11c<sup>highMac-1<sup>neg/low</sup> AMΦs (subpopulation Rii) and an extra subpopulation of CD11c<sup>highMac-1<sup>pos</sup> AMΦs (subpopulation Ri), and no lung monocyte involvement. Both subpopulations were Ly6cneg, a characteristic of nonrecruited (subpopulation Rii), and no lung monocyte involvement. Both subpopulations were Ly6cneg, a characteristic of nonrecruited AMΦs (Fig. 4C). Interestingly, SHIP-1<sup>−/−</sup> AMΦs were more autofluorescent than C57BL/6 AMΦs, with a more diffuse increased cytoplasmic signal observed (Fig. 4D). SHIP-1<sup>−/−</sup> Mac-1<sup>pos</sup> AMΦs (subpopulation Ri) were larger, highly vacuolated, and often multinucleated compared with C57BL/6 and SHIP-1<sup>−/−</sup> Mac-1<sup>neg/low</sup> AMΦs (Fig. 4E). Cell sorting showed significant upregulation of iNOS and Arg I in both SHIP-1<sup>−/−</sup> AMΦ subpopulations (Fig. 4F). Interestingly, high MMP-12 expression segregated with only subpopulation Rii (Fig. 4F).

Acute cigarette smoke exposure was studied alongside C57BL/6 SHIP-1<sup>−/−</sup> mice as a negative control for the specificity of AMΦ subpopulation formation in pathological disease. Although cigarette smoke is the major epidemiological cause of COPD and lung cancer, models of acute cigarette exposure do not model the development of chronic lung disease (27), although neutrophilic lung inflammation is observed (22). In C57BL/6 mice, acute cigarette smoke exposure did not induce AMΦ Mac-1 upregulation despite an increase in BAL neutrophil and MΦ numbers, increased AMΦ autofluorescence, and AMΦ granularity. This further indicates that AMΦ subpopulations may correlate with selective immunological and pathological processes, rather than nonspecifically in all inflammatory lung conditions (Fig. 4A–D).

AMΦ subpopulations are disease dependent in SHIP-1<sup>−/−</sup> mice

Chronic lung disease is background dependent in SHIP-1<sup>−/−</sup> mice (21); with lung pathology manifest in the C57BL/6 but not BALB/c background at 12 wk (Fig. 5A). Although deletion of SHIP-1 produces a rare subpopulation of Mac-1<sup>pos</sup> AMΦs with dendritic-like processes in BALB/c mice, large, morphologically activated Mac-1<sup>pos</sup> AMΦs are only a characteristic of C57BL/6 SHIP-1<sup>−/−</sup> mice (Fig. 5B, 5C), thus tracking with lung disease rather than intrinsic SHIP-1 deletion itself.

AMΦ subpopulations track with spontaneous lung disease induction

We further followed AMΦ subpopulation deregulation using a novel model of spontaneous lung disease induction. CD45.1<sup>+</sup> BL6.SJL-Ptprc<sup>−/−</sup> chimeric mice were transplanted with CD45.2<sup>+</sup> C57BL/6 or SHIP-1<sup>−/−</sup> BM to study whether hematopoietic SHIP-1 deletion was sufficient to spontaneously induce lung disease. We found residential AMΦ replacement varies between paper and wood-chip bedding, with wood-chip bedding greatly exacerbating residential AMΦ replacement. To ensure complete replacement by donor AMΦs, we specifically housed all disease induction experiments on wood-chip bedding, to ascertain that any lung pathology would be a result of recipient and not remaining donor AMΦs. Six weeks posttransplant, residential AMΦs replacement was now 80–85% CD45.1<sup>+</sup> in C57BL/6 chimeras and >99% CD45.1<sup>+</sup> in SHIP-1<sup>−/−</sup> chimeras, with no changes in the residential CD11c<sup>highMac-1<sup>neg/low</sup></sup> phenotype or lung pathology observed (Fig. 5D, 5E). Eight to 10 mo posttransplantation, when symptoms of illness were first identified, spontaneous granulomatous lung disease was observed in all SHIP-1<sup>−/−</sup> but not C57BL/6 chimeras, and accompanied by Mac-1<sup>pos</sup> AMΦ subpopulation formation (Fig. 5D, 5E). Interestingly, SHIP-1<sup>−/−</sup> chimeras did not develop the same lung disease phenotype as C57BL/6 SHIP-1<sup>−/−</sup> mice, possibly because of their retention of a subset of CD45.1<sup>+</sup> SHIP-1<sup>−/−</sup> blood lymphocytes (Supplemental Fig. 1D).

Discussion

In this article, to our knowledge, we present the first findings segregating residential MΦ heterogeneity into distinct subpopulations during acute lung inflammation and chronic inflammatory lung disease. Currently, identification of putative tissue MΦ subsets is of great interest, because it is now believed that an imbalance of homeostatic versus pathogenic MΦ subsets may be driving the pathogenesis of inflammation and disease (1). Recently, differential F4/80 and CD11c surface expression has been used to discriminate distinct MΦ and dendritic cell subsets in the colon, which shift in balance during gut homeostasis and induced inflammatory disease (28). In lung, although significant advances have been made in the characterization of dendritic cell subsets (29), inflammatory lung monocytes and blood-derived exudate MΦs (14), the intense autofluorescence of residential AMΦs has rendered the global study of lung MΦ heterogeneity highly difficult. In this study, we used Mac-1 and CD11c surface expression as a clear segregator of residential as well as emigrating monocyte and lung MΦ subsets.

In healthy lungs at rest, CD11c<sup>highMac-1<sup>neg/low</sup></sup> residential AMΦs (subpopulation R) maintain immunological homeostasis through constant removal of innocuous foreign Ags found in the local lung microenvironment (30) and through suppression of inflammatory T cell responses through regulation of pulmonary DC activity and migration (10). LPS challenge orchestrates two MΦ-related changes in alveolar airspaces. First, a subpopulation of residential AMΦs upregulate Mac-1 expression (subpopulation...
FIGURE 5. Mac-1\(^{+}\) AMΦ subpopulation tracks with lung disease independent of SHIP-1 deletion. (A) MT-stained lungs from 12-wk-old BALB/c, BALB/c SHIP-1\(^{-/-}\), C57BL/6, and C57BL/6 SHIP-1\(^{-/-}\) mice. Red arrows indicate myeloid infiltrates in C57BL/6 SHIP-1\(^{-/-}\) alveolar airspaces. Scale bar, 20 \(\mu\)m. (B) Flow cytometry of Mac-1 versus CD11c expression of BAL MΦs. Representative of three independent experiments; \(n = 3–6\). (C) Cytospins of sorted BAL MΦ subpopulations. Representative of two independent sorts. Scale bar, 20 \(\mu\)m. (D) MT-stained lungs from respective chimeric mice. Red arrows indicate myeloid infiltrates. Scale bar, 100 \(\mu\)m. (E) Mac-1 versus CD11c, and CD45.2 versus CD45.1 expression of 6-wk-old chimeric mice. Representative of two independent experiments; \(n = 3–8\) mice. (F) Mac-1 versus CD11c, and CD45.2 versus CD45.1 expression of 10-mo-old chimeric mice. Representative of two independent experiments; \(n = 3–8\) mice.
I). Shortly afterward, inflammatory blood monocytes (subpopulation M) are recruited into alveolar airspaces where they mediate bactericidal activity directly through phagocytosis and NO production, and indirectly through the amplification of the local inflammatory response. Although recruitment of inflammatory lung monocytes has been well studied (11, 13, 31), to our knowledge, we are the first to identify their simultaneous expression of both M1- and M2-like MΦ markers ex vivo. This is significant because the current doctrine tends to regard M1 and M2 MΦs as differentially polarized MΦ subtypes that mediate distinct functions in the tissue microenvironment (32, 33), trending the identification of a M2 MΦ based solely on its expression of M2 markers such as Arg I and IL-10. Because lung MΦs exist in a microenvironment that is dynamic and complex, ex vivo characterization of functionally distinct subpopulations using flow cytometry may allow us to better understand the role of AMΦs in homeostasis and disease.

Monocytes are key effector cells in inflammation and host defense (14). Interestingly, we also discriminated key differences in the lung monocyte subpopulation postinfection with Mem71 influenza A virus compared with LPS treatment. First, LPS-mediated acute lung inflammation is resolved by day 10, whereas Mem71 infection triggers a longer lasting complex MΦ recruitment circuit in lung. Second, infiltrating monocytes are more dominant in Mem71-infected compared with LPS-treated mice, with the subpopulation Mv monocytes expressing higher levels of iNOS, IL-6, Arg I, and IL-10. Mem71 is a type A influenza strain of intermediate virulence that, given at sublethal dosages, induces a lung inflammatory response that peaks at day 3 and is resolved by day 10 (34). In contrast, infection with high-fatality influenza A strains (i.e., avian H5N1/97) leads to patient death from acute respiratory distress and multiple organ dysfunction, postulated to be caused by aberrant cytokine production from virus-exposed lung MΦs (5, 35). Interestingly, CCR2+/− mice infected with the highly virulent PR8 influenza A strain virus have decreased lung monocyte recruitment and are protected against morbidity and mortality independent of virus clearance efficiency (6, 14), clearly highlighting the significant contributions of lung monocytes in acute lung disease. In our model of nonlethal influenza infection, we observed the highest levels of iNOS and IL-6, but also Arg I and IL-10, in subpopulation Mv. Arg I suppresses NO production through competition for arginine substrate with iNOS (36), and its simultaneous upregulation may help regulate NO levels to prevent NO-mediated pneumonitis and mortality after influenza infection (37). IL-10 deactivates MΦ proinflammatory cytokine production in vitro (38). Although IL-10 production by effector T cells is implicated in the suppression of influenza-induced inflammation and lethality (39), we show that the CD11c−/low/Mac-1high Mv subpopulation already produces IL-10 during the peak innate response. Thus, we postulate that lung monocytes may exhibit both antiinflammatory and proinflammatory mechanisms in vivo to protect from influenza-induced cytokine dysfunction and mortality. In particular, it will be greatly interesting to examine whether influenza pathology occurs alongside a global imbalance of the lung MΦ subpopulations or a shift in the M2 < M1 simultaneous characteristics of recruited lung monocytes.

Because it has been known for some time that increased AMΦ numbers clinically correlate with COPD severity (16, 40), animal models of chronic inflammatory lung disease are of great interest. Although large vacuolated AMΦs are found in almost all animal models of COPD (7, 17, 41, 42), no single pathological AMΦ subpopulation has yet been identified or associated with disease pathogenesis. In this article, we present the presence of a subpopulation of constitutively Mac-1high residential AMΦs in C57BL/6 SHIP-1/−/− mice that spontaneously develop COPD-like lung disease. Our findings implicate this Mac-1high AMΦ subset as the agents of disease rather than SHIP-1 deletion itself, because BALB/c SHIP-1/−/− mice that lack disease do not have this prominent Mac-1high AMΦ subpopulation. Interestingly, high MMP-12 expression tracks specifically with the Mac-1high AMΦ subpopulation in C57BL/6 SHIP-1/−/− mice. Downstream MMP-12 upregulation is a common feature of all COPD animal models (7, 17, 41, 42), implicating its role as a major COPD effector. Clinically, a minor SNP allele in MMP-12 that reduces its protease activity is protective against COPD onset in smokers (43). Because MMP-12 expression is also transiently upregulated in Mac-1high AMΦs after LPS treatment, we postulate that deregulation of SHIP-1 signaling in a susceptible genetic background creates spontaneous AMΦ heterogeneity wherein chronic AMΦ Mac-1, and hence MMP-12, expression may be responsible for the lung disease pathogenesis.

In summary, we have used a novel flow cytometry approach to identify and characterize AMΦ subpopulation heterogeneity in both acute lung inflammation and chronic lung disease. We report Mac-1 as a marker of activated residential AMΦs and show that ex vivo lung MΦs adopt both M1 and M2-like characteristics during acute inflammation and disease. Specifically, we show that AMΦ subpopulations are a feature of and track with the development of chronic inflammatory lung disease. This study paves the way for elucidating the role of AMΦ subpopulations in lung disease pathogenesis, and the possibility of selectively targeting pathological AMΦ subpopulations to attenuate lung disease.

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Disclosures
The authors have no financial conflicts of interest.

References


SUPPLEMENTARY FIGURE 1. A, Non-differential expression of M1 (CD14 and CD40) and M2 surface markers (CD23, CD36 and CD124) in AMφs from control and LPS-treated C57BL/6 mice. Positive staining in black, and staining with non-binding control Abs in grey; representative of two independent experiments, n = 4. B, Enlarged cytospins of sorted lung Me and Mv monocyte-like subpopulations from LPS-treated and Mem71-infected mice respectively from Fig. 2D compared with blood monocytes (open arrows) and neutrophils (solid arrows) from control C57BL/6 mice, scale bar = 20 μm. C, FSC vs. SSC (top panels), and CD45.2 vs. CD45.1 expression (bottom panels) on BAL cells of C57BL/6 vs. CD45.2 chimeric mice four weeks post-reconstitution; representative of two independent experiments, n = 4. B, CD45.2 vs. CD45.1 expression of Mac-1pos Ly6glow blood cells (i.e. all myeloid cells) in C57BL/6 and CD45.2 chimeric mice; representative of two independent experiments, n = 4. D, CD45.2 vs. CD45.1 expression of blood leukocytes in 10 month old C57BL/6 and SHIP-1-/- chimeric mice; representative of n = 3-8 mice; 2 independent experiments.
Supplementary Figure 1

A

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B

LPS BAL Me  
VIRUS BAL Mv  
C57BL/6 Blood

C

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BAL cells  
CD45.1 = donor  
CD45.2 = recipient

D

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Blood  
CD45.1 = recipient  
CD45.2 = donor

E

- C57 BM  
- SHIP-1 BM  
- C57 SHIP-1

Blood  
CD45.1 = recipient  
CD45.2 = donor