Distinct developmental pathways from blood monocytes generate human lung macrophage diversity

Graphical Abstract

Highlights

- A developmental map of human lung macrophages from blood monocytes in vivo
- Extravasating CD14+ monocytes give rise to alveolar and interstitial macrophages
- Identification of CD14+HLA-DRhi lung monocytes as intermediate differentiation stage
- Pulmonary intravascular macrophages originate from CD16+ blood monocytes

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In Brief

Tissue-resident macrophages maintain healthy organ function, but the ontogeny of human macrophages is largely unknown. Using humanized mice and single-cell RNA sequencing, Evren et al. uncover the migration and differentiation of blood monocytes into distinct populations of human lung macrophages in vivo.
Distinct developmental pathways from blood monocytes generate human lung macrophage diversity

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SUMMARY

The study of human macrophages and their ontogeny is an important unresolved issue. Here, we use a humanized mouse model expressing human cytokines to dissect the development of lung macrophages from human hematopoiesis in vivo. Human CD34+ hematopoietic stem and progenitor cells (HSPCs) generated three macrophage populations, occupying separate anatomical niches in the lung. Intravascular cell labeling, cell transplantation, and fate-mapping studies established that classical CD14+ blood monocytes derived from HSPCs migrated into lung tissue and gave rise to human interstitial and alveolar macrophages. In contrast, non-classical CD16+ blood monocytes preferentially generated macrophages resident in the lung vasculature (pulmonary intravascular macrophages). Finally, single-cell RNA sequencing defined intermediate differentiation stages in human lung macrophage development from blood monocytes. This study identifies distinct developmental pathways from circulating monocytes to lung macrophages and reveals how cellular origin contributes to human macrophage identity, diversity, and localization in vivo.

INTRODUCTION

The lung is a vital organ exposed to airborne challenges that are first encountered by tissue-resident macrophages. At least two functionally distinct macrophage populations residing in different anatomical niches exist in the lung (Evren et al., 2020; Garbi and Lambrecht, 2017; Russell and Bell, 2014; Joshi et al., 2018; Kopf et al., 2015; Liegeois et al., 2018; Lloyd and Marsland, 2017; Putter et al., 2019). Alveolar macrophages catabolize lung surfactant, phagocytose-inhaled material, and orchestrate immune responses against airborne microbes. Lack of alveolar macrophages causes pulmonary alveolar proteinosis (PAP), an inflammatory syndrome due to the accumulation of surfactant in the alveoli (Trapnell et al., 2019). Interstitial macrophages located within the space between the lung epithelium and the vasculature perform tissue remodeling and contribute to barrier immunity through antigen presentation.

Macrophages originate from either embryonic precursors or from blood monocytes arising from hematopoietic stem cells in the bone marrow (Epelman et al., 2014; Ginhoux and Guilliams, 2016; Perdiguero and Geissmann, 2016; Varol et al., 2015). Mouse alveolar macrophages develop from fetal monocytes after birth in response to granulocyte-macrophage colony-stimulating factor (GM-CSF) that induces the master transcription factor peroxisome proliferator-activated receptor gamma (PPARγ) (Guilliams et al., 2013; Li et al., 2020; Schneider et al., 2014; Yu et al., 2017). In steady-state, mouse alveolar macrophages are largely maintained through local self-renewal (Genet et al., 2014; Sieweke and Allen, 2013; Soucie et al., 2016) independently of circulating monocytes (Guilliams et al., 2013; Hashimoto et al., 2013; Yona et al., 2013). However, blood monocytes derived from hematopoietic stem cells increasingly contribute to the alveolar macrophage compartment in older mice (Gomez Perdiguero et al., 2015; Liu et al., 2019). Mouse interstitial macrophages originate mainly from adult hematopoiesis (Sabatel et al., 2017; Tan and Krasnow, 2016) and are slowly replaced by circulating monocytes (Chakarov et al., 2019; Gribings et al., 2017; Schyns et al., 2019).
Figure 1. All types of lung monocytes and macrophages originate from human HSPCs in MISTRG mice
(A) Engraftment of MISTRG mice with human CD34+ HSPCs. Newborn MISTRG mice transplanted with CD34+ cells from cord blood were analyzed 8 weeks post-transplantation.

(B) Bronchoalveolar lavage

(C) Lung tissue

(D) CD45+CD11b+CD64+CD14+CD16-

(E) MISTRG

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In contrast, due to the lack of suitable in vivo models, the ontogeny of human lung macrophages remains poorly understood. Lung transplantation studies indicate that human interstitial and alveolar macrophages mainly arise from recruited hematopoietic precursors, likely circulating monocytes (Bittmann et al., 2001; Byrne et al., 2020; Eguluz-Gracia et al., 2016; Kjellström et al., 2000; Nayak et al., 2016). This is presumably because humans, in contrast to laboratory mice housed in specific-pathogen-free conditions, are continuously exposed to respiratory pathogens. Overall, these studies highlight the importance of monocyte-derived lung macrophages in humans, yet their developmental paths and cellular precursors remain undefined.

Circulating monocytes can enter tissues and differentiate into tissue-resident macrophages (Guillas et al., 2018). In humans, the two main subsets of blood monocytes are classical CD14+CD16− monocytes and non-classical CD14+CD16+ monocytes that patrol blood vessels (Cros et al., 2010; Ziegler-Heitbrock, 2015). According to the current model, classical monocytes either extravasate into tissues or differentiate into patrolling CD14+CD16+ monocytes via CD14+CD16+ intermediate monocytes (Patel et al., 2017a; Tak et al., 2017). However, the in vivo macrophage potential of human blood monocyte subsets has not been determined.

Investigating human macrophages in vivo represents a considerable challenge. Therefore, we developed a humanized mouse model, named “MISTRG” (Rongvaux et al., 2014), expressing factors that are critical for human hematopoiensis through genetic targeting, thereby overcoming the lack of cross-reactivity between mouse factors and human receptors (Alisjahbana et al., 2020; Rongvaux et al., 2013; Willinger et al., 2011a). Apart from macrophage colony-stimulating factor (M-CSF) and GM-CSF, two cytokines essential for macrophage development (Epelman et al., 2014; Lavin et al., 2015), MISTRG mice express the human cytokines interleukin-3 (IL-3) and thrombopoietin (TPO) in the immunodeficient Rag2−/−Il2rg−/− background, as well as signal-regulatory protein alpha (SIRPα) to establish mouse-to-human phagocytic tolerance (Rongvaux et al., 2014). This “genetic pre-conditioning” creates a highly permissive environment for human hematopoiensis in the mouse host (Rongvaux et al., 2014; Willinger et al., 2011a). MISTRG mice support the reconstitution of a human blood monocyte and lung macrophage compartment after transplantation with human CD34+ hematopoietic stem and progenitor cells (HSPCs) (Rongvaux et al., 2014; Willinger et al., 2011b). Therefore, MISTRG mice offer the opportunity to study human macrophages within their surrounding tissue microenvironments, thereby allowing in vivo studies that are possible in humans.

Here, we define human macrophage ontogeny from precursor to mature lung macrophage using our humanized mouse model in combination with progenitor transplantation, fate mapping, intravascular cell labeling, and single-cell RNA sequencing (scRNA-seq). We identify classical CD14+CD16− monocytes as circulating precursors of lung tissue monocytes as well as interstitial and alveolar macrophages. In contrast, non-classical CD14+CD16− monocytes were confined to the lung vasculature and gave rise to a distinct population of pulmonary intravascular macrophages. Our findings reveal the impact of cellular origin on human macrophage heterogeneity, localization, and functional specification.

RESULTS

HSPCs give rise to all types of human lung monocytes and macrophages in MISTRG mice

Human CD68+ lung macrophages develop in MISTRG mice transplanted with fetal, neonatal, or adult CD34+ cells (Rongvaux et al., 2014; Saito et al., 2016; Sippel et al., 2019; Willinger et al., 2011b). We used recently assigned cell-surface phenotypes (Bharat et al., 2016; Desch et al., 2016; Yu et al., 2016) to more precisely define different human lung macrophage populations in MISTRG mice. Newborn MISTRG mice were transplanted with human CD34+ HSPCs from umbilical cord blood (without pre-conditioning to avoid irradiation-induced tissue damage) and analyzed 8 weeks post-transplantation (Figure 1A; see Figures S1A and S1B for gating strategy). The main cell population found in the bronchoalveolar lavage (BAL) fluid of engrafted MISTRG mice (Figure 1B), were large autofluorescent human CD45+HLA-DR−CD11b+ cells that expressed cell-surface proteins characteristic of human alveolar macrophages (Bharat et al., 2016; Yu et al., 2016), such as CD206 (mannose receptor), CD169 (Siglec-1), and macrophage receptor with collagenous structure (MARCO). Human alveolar macrophages derived from CD34+ HSPCs in MISTRG mice had a similar phenotype to their counterparts found in the BAL fluid of healthy human donors (Figure 1B). CD206+CD169+ macrophages were also present in digested lung tissue of MISTRG mice transplanted with human CD34+ HSPCs and in the human lung (Figure 1C). In addition, lung tissue harbored a population of human CD45+CD11b−CD64+CD14+CD16− extravascular cells that were smaller than alveolar macrophages and had a CD206+HLA-DR−CD169− phenotype (Figures 1D, S1C, and S1D), characteristic of human interstitial lung macrophages (Chakarov et al., 2019). A third population of CD45+HLA-DR−CD11b+ cells lacked expression of CD206 and CD169, consisting mainly of CD14+ lung monocytes (Figure 1C), similar to their equivalent in the human lung (Baharom et al., 2016; Bharat et al., 2016; Desch et al., 2016; Yu et al., 2016). Immunofluorescence microscopy and immunohistochemistry of lung sections confirmed the presence of human macrophages expressing CD206, MARCO, CD169, CD163, CD68, and CD14 in MISTRG mice transplanted with human CD34+ cells (Figures S2A and S2B). Human lung macrophages

(B and C) Flow cytometry analysis of human macrophages in bronchoalveolar lavage (BAL) fluid (B) and lung tissue (C) from HSPC-engrafted MISTRG mice (n = 8) and from healthy human donors (n = 3). Cells were pre-gated as in Figures S1A and S1B.

(D) Flow cytometry of human interstitial macrophages in lung tissue from healthy donors (n = 3) and HSPC-engrafted MISTRG mice (n = 11). Cells were pre-gated as in Figure S1C.

(E) Amounts of total protein in BAL fluid of 2-month-old MISTRG mice that were either not engrafted or engrafted as newborns with human CD34+ HSPC (n = 4–10).

Data are represented as mean ± SEM. Data are representative of three experiments. See also Figures S1–S3. (A) was adapted from Servier Medical Art.
Figure 2. Kinetics of human lung macrophage development in HSPC-engrafted MISTRG mice
(A–C) Frequency and number of human lung monocytes (B) and macrophages (C) in MISTRG mice after transplantation with human CD34+ HSPCs (n = 7–21). Cells were pre-gated as in Figure S1B. Lin, lineage (CD3, CD19, CD56, CD66abce, NKp46).

(D) Human CD34+ HSPCs after intranasal administration of LPS or Poly I:C

(E) Frequency of CD206+CD169+ in HLA-DR+:CD11b+ population

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originating from CD34+ HSPCs were functional since they rescued PAP syndrome (Figure 1E), which develops in non-transplanted homozygous MISTRG mice due to the lack of mouse alveolar macrophages (Figure S3) (Willinger et al., 2011b). Furthermore, human lung myeloid cells phagocytosed airborne E. coli bioparticles and upregulated the pro-inflammatory cytokines IL6 and TNF after intranasal lipopolysaccharide (LPS) administration (Figures S2C and S2D). Taken together, we demonstrate that MISTRG mice transplanted with human CD34+ HSPCs harbor lung monocytes, interstitial macrophages, and alveolar macrophages that are phenotypically similar to their counterparts in the human lung.

**CD14**^+**CD16**^− monocytes highly express GM-CSF receptor and appear early in the lung

We next asked which blood monocyte subset gives rise to human lung macrophages in MISTRG mice transplanted with HSPCs. We hypothesized that the monocyctic precursor of human alveolar macrophages should be highly responsive to the key cytokine GM-CSF. To test this hypothesis, we determined GM-CSF receptor expression on the different blood monocyte subsets, both in healthy human donors and in MISTRG mice engrafted with CD34^+ cells. All blood monocytes expressed GM-CSF receptor alpha (CD116) on their cell surface, with classical CD14^+CD16^− monocytes having the highest expression (Figure S4A). We then predicted that macrophage precursors colonize the “empty” lung in MISTRG mice before developing into lung macrophages. To test this prediction, we examined when HSPC-derived human monocytes and macrophages populate the lung after transplantation of newborn MISTRG mice with CD34^+ cells (Figure 2A). Flow cytometry (see Figure S1B for gating strategy) revealed that classical CD14^+CD16^− monocytes were present in the lung at 3 weeks, i.e., earlier than the other monocyte subsets (Figure 2B). Furthermore, CD206^+CD169^− macrophages were present before CD206^+CD169^+ macrophages developed and are therefore likely the monocyte-derived precursor of CD206^+CD169^+ alveolar macrophages (Figure 2C). Lung inflammation induced by Poly(I:C) or LPS accelerated the differentiation of human CD206^+CD169^+ macrophages in MISTRG mice (Figure 2E).

**Circulating CD14**^+**CD16**^− monocytes migrate into lung tissue and give rise to human lung macrophages in vivo

We next reasoned that macrophage differentiation from monocytes is linked to a stepwise migration from the circulation into the different anatomical compartments of the lung, where macrophage precursors are exposed to local tissue-instructive factors. Consequently, we asked whether human monocytes in the MISTRG lung were able to extravasate from the blood into the lung tissue. To distinguish vascular from tissue monocytes in MISTRG mice engrafted with CD34^+ HSPCs, we performed labeling of intravascular human hematopoietic cells by the intravenous injection of a phycoerythrin (PE)-conjugated anti-human CD45 antibody (Figure 3A). As expected, all nucleated human hematopoietic cells in blood were labeled, whereas CD45^+ cells from the BAL were protected from antibody labeling due to their extravascular location (Figures S4B and S4C). Intravascular labeling revealed that CD14^+CD16^− monocytes were the main monocyte subset located outside of blood vessels in the lung (Figures 3B and 3C), consistent with active extravasation into lung tissue.

Based on these findings, we hypothesized that CD14^+CD16^− blood monocytes are the progenitor of human lung macrophages. To test this hypothesis, we adoptively transferred purified blood monocyte subsets into adult MISTRG mice (not transplanted with human CD34^+ cells) (Figure 3D). Intravenously injected CD14^+CD16^− monocytes, but not CD14^+CD16^+ monocytes, generated human lung macrophages with an alveolar macrophage phenotype (CD45^HI-LA-DR^−CD11b^−CD206^+CD169^+) 3 weeks post-transfer (Figure 3E). Collectively, our data demonstrate that classical CD14^+CD16^− blood monocytes are able to extravasate and migrate into the alveolar niche, where they differentiate into lung macrophages.

**Fate mapping identifies CD14**^+**CD16**^− blood monocytes as precursors of HSPC-derived human lung macrophages

To further confirm a precursor-product relationship between blood monocytes and lung macrophages, we performed fate mapping of human monocytes in MISTRG mice engrafted with CD34^+ HSPCs (Figure 4A), adopting a previously used method to fate-map mouse monocytes (Ginhoux et al., 2006; Tacke et al., 2006). CD16^− blood monocytes were specifically labeled by the intravenous injection of HSPC-engrafted MISTRG mice with fluorescent latex beads (Figures 4A–4C), which were first captured by CD14^+CD16^− monocytes that then differentiate into CD16^+ monocytes (Patel et al., 2017a). To label CD14^+CD16^− blood monocytes, circulating monocytes were first depleted by intravenous clodronate treatment 1 day before bead injection (Figure 4A). In this case, CD14^+CD16^− blood monocytes, corresponding to recently released monocytes from the bone marrow, but not CD16^+ monocytes took up the beads (Figures 4B and 4C). Flow cytometry of fate-mapped cells in the lung 1 week after bead injection showed that CD14^+CD16^− blood monocytes more efficiently generated lung monocytes and CD206^+CD169^+ lung macrophages than CD16^+ blood monocytes (Figures 4D and 4E). The average frequency of CD206^+CD169^+ lung macrophages was quite low, likely because 1 week is not long enough for the full differentiation of CD206^+CD169^+ macrophages, derived from bead-labeled CD14^+CD16^− blood monocytes, into CD206^+CD169^+ lung macrophages to occur. We conclude that HSPC-derived human lung macrophages arise mainly from CD14^+CD16^− blood monocytes.

**scRNA-seq reveals the heterogeneity of HSPC-derived human lung monocytes and macrophages**

Next, we performed scRNA-seq of human CD45^+CD11b^HI-LA-DR^+ myeloid cells isolated from the lungs of MISTRG mice to fully

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(D and E) Flow cytometry analysis of human lung macrophage development in HSPC-engrafted MISTRG mice in the presence of local inflammation (n = 3–6). LPS, lipopolysaccharide.

Data are represented as mean ± SEM. Data are representative of or combined from two (D and E) or three (A–C) experiments. See also Figures S1 and S4A. (A) and (D) were adapted from Servier Medical Art.
Figure 3. CD14+CD16– blood monocytes extravasate into lung tissue and differentiate into human lung macrophages in vivo

(A) Intravascular labeling of human CD45+ hematopoietic cells in MISTRG mice engrafted with CD34+ HSPCs by intravenous (IV) injection of anti-human CD45-PE antibody.

(B) Flow cytometry analysis of intravascular and in tissue localization of CD14+CD16– monocytes.

(C) Statistical analysis showing the percentage of CD14+CD16– monocytes in tissue.

(D) Adoptive monocyte transfer from healthy donors to non-engrafted MISTRG mice.

(E) Flow cytometry analysis of CD11b and HLA-DR expression in lung cells.

(p = 0.0004, p = 0.0002, p = 0.7910)
(p = 0.0163)
define the heterogeneity of HSPC-derived human lung monocytes and macrophages in vivo (Figure 5A). Based on our kinetic experiments (Figures 2A–2C), we chose day 21 and 36 after transplantation with human CD34+ HSPCs to capture both potential macrophage progenitors and different stages of mature cells. Unsupervised clustering of 28,207 cells by UMAP revealed 10 transcriptionally distinct cell clusters at day 36 (Figures 5B and S5A; Table S1). We first focused on populations corresponding to monocyte subsets in the lung (Figures 5B and S5B). Cluster 3 (CD14+, VCAN, LYZ, S100A8, S100A9, S100A12) was identified as CD14+CD16- monocytes, whereas cluster 2 (FCGR3A, LST1, AIF1, CTSS, CD52) corresponded to CD14+CD16+ monocytes, similar to classical and non-classical monocytes in human blood (Villani et al., 2017) and lung (Lavin et al., 2017; Zilionis et al., 2019). Cluster 5 expressed genes involved in oxidative phosphorylation (MT-CYB, MT-CO1-3, MT-ND2-5, MT-ATP6, UCP2), suggesting that this cluster constitutes a subset of CD14+ monocytes with a distinctive cellular metabolism. Cluster 0 expressed HLA-DR, HLA-DR, HLA-DQ, and other transcripts (MS4A6A, LGALS2, CPVL) characteristic of CD14+HLA-DR+ monocytes (Reyes et al., 2020; Schmidt et al., 2014; Wong et al., 2011; Zawada et al., 2011). Finally, cluster 1 expressed CTNNB1, encoding β-catenin, an integral part of the WNT pathway, which promotes monocyte-to-lymphoid macrophage differentiation in mice (Sennello et al., 2017). A similar monocyte subset was very recently described in human blood (Reyes et al., 2020) and may represent quiescent CD14+ monocytes.

We next focused on lung macrophages, which segregated into three distinct clusters (Figures 5B and S5C). Cells in cluster 4 expressed the master transcription factor PPARγ, genes related to lipid metabolism (APOC1, APOE, ALOX5AP, FABPS) and other genes (LAMP1, OCHFR, GPNNB, INHBA) characteristic of human alveolar macrophages (Desch et al., 2016; Morse et al., 2019; Pateli et al., 2017b; Vieira Braga et al., 2019). Cluster 6 macrophages had high expression of genes encoding chemoattractants (CCL2, CCL4, CCL8, CXCL10, CXCL11) and the pro-inflammatory cytokine IL1B, similar to mouse interstitial macrophages (McCubrey et al., 2018; Sañti et al., 2020; Schyns et al., 2019). Furthermore, cluster 6 macrophages expressed a number of interferon-induced genes (ISG15, ISG20, IFITM1, IFIT3, IFI6). Overall, this suggested that cluster 6 macrophages contribute to human intestinal lung macrophages. Finally, cluster 7 macrophages were distinguished by the expression of complement-related genes (C1QA, C1QB, C1QC, C3AR1, CFD) and genes involved in iron metabolism (IFT1, FTH1, ACP5). Altogether, our scRNA-seq data confirm that diverse human lung macrophage populations are generated from HSPCs in vivo.

**Extravascular HLA-DRhi monocytes represent a cellular intermediate in human lung macrophage development**

To further infer lineage relationships between human monocytes and lung macrophages, we performed developmental trajectory analysis with Slingshot (Street et al., 2018). The trajectory from CD14+CD16+ monocytes first proceeded via OxPhos monocytes (cluster 5) toward HLA-DRhi monocytes (cluster 0) (Figure 5C). HLA-DRhi monocytes occupied a central position, with separate trajectories directed toward non-classical CD14loCD16+ monocytes (cluster 2), CTNNB1-monocytes (cluster 1), as well as interstitial (cluster 6) and alveolar macrophages (cluster 4). The trajectory from CD14loCD16+ monocytes further pointed toward C1Q macrophages (cluster 7). These data further support the notion that CD14+CD16+ monocytes, via HLA-DRhi monocytes, are the main precursor of human HSPC-derived interstitial and alveolar macrophages. The trajectory analysis also suggested that CD14loCD16+ monocytes may give rise to a discrete type of human lung macrophages (C1Q macrophages).

HLA-DRhi monocytes (cluster 0) expressed the tissue residency marker CD69 (Table S1) and genes (MEF2C, S100A10) promoting monopoiesis and tissue invasion (O’Connell et al., 2010; Phipps et al., 2011; Schüler et al., 2008; Schwieger et al., 2009). We therefore hypothesized that HLA-DRhi monocytes actively migrate into lung tissue. Consistent with our hypothesis, we found a population of extravascular CD14+ HLA-DRhi monocytes in the lung at 4 weeks post-transplantation with CD34+ cells (Figure 5D), when monocyte-to-lymphoid macrophage differentiation occurs (Figure 2C). Combined these data indicate a stepwise differentiation process from circulating CD14+ monocytes, via extravasating CD14+HLA-DR+ monocytes and immature CD206+CD169+ macrophages, toward CD206+CD169+ alveolar macrophages.

To further elucidate the early events of human lung macrophage differentiation from monocytes, we also performed scRNA-seq of human lung myeloid cells at day 21 after HSPC transplantation. Similar to day 36, three monocyte clusters were present in the lung at day 21: classical CD14+ monocytes (cluster 1), HLA-DRhi monocytes (cluster 0), and non-classical CD16+ monocytes (cluster 3) (Figure 5E; Table S2). Furthermore, clusters resembling interstitial (cluster 4) and alveolar macrophages (cluster 2 and 8) could be detected at day 21. Analysis at day 21 also revealed a macrophage cluster (cluster 6) that was not found at day 36. Cluster 6 macrophages were characterized by the expression of ribosomal, ubiquitin, and proteasome genes (Table S2), suggesting active protein synthesis and turnover. Cluster 6 macrophages also preferentially expressed the transcription factor CEBPB that is required for alveolar macrophage development in mice (Cain et al., 2013). Furthermore, CBX7 (part of the polycomb repressive complex) was one of the top genes expressed by cluster 6 macrophages. CBX7 regulates HSC self-renewal and its downregulation induces macrophage differentiation (Jung et al., 2019; Klauke et al., 2013). Overall, these findings suggest that cluster 6 represents a transitional macrophage stage before terminal differentiation into mature lung macrophages. Finally, we observed expression of IL7R by developing lung macrophages (Figure 5F; Table S2), consistent with the recently described role of IL-7Rα in the
A) Fate-mapping of CD16+ monocytes

HSPC engrafted MISTRG (5 weeks old)

Blood

CD14

CD16

Conversion

Blood

Bleeding

Sacrifice

D1

D3

D7

IV injection of PE beads

IV injection of clodronate of PE beads

B) Blood D3 CD16+ fate-mapping

CD16

CD14

CD14+ fate-mapping

CD16

CD14

CD206

CD169

CD206 CD169 macrophages

D) Lung D7

No beads

CD14+ fate-mapping

CD16+ fate-mapping

E) Blood monocytes D7

Fate-mapped (%) vs CD14+ fate-mapping vs CD16+ fate-mapping

Lung D7

Fate-mapped (%) vs CD206 CD169 macrophages

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development of tissue-resident macrophages in mice (Leung et al., 2019).

Identification of human intravascular lung macrophages in HSPC-engrafted MISTRG mice

Next, we aimed to better characterize C1Q macrophages found in cluster 7 at day 36. Apart from complement genes, cluster 7 macrophages expressed genes involved in red blood cell degradation and iron metabolism (Table S1). This led us to hypothesize that cluster 7 may contain macrophages with an intravascular localization. Consistent with our hypothesis, we detected a population of human CD206+CD169+ macrophages that was labeled by intravenous CD45-PE antibody in the lungs of MISTRG mice engrafted with human CD34+ HSPCs (Figure 6A). This suggested that some human lung macrophages resided in an intravascular niche, where they were exposed to intravenously injected antibody. CD206+CD169+ lung macrophages labeled by intravenous CD45-PE antibody were distinct from intravascular CD206+CD169+ monocytes and extravascular CD206+CD169+ macrophages based on their intermediate size and granularity (Figure 6A). CD206+CD169+ macrophages labeled by intravenous CD45-PE antibody expressed CD163 (Figure 6A), the scavenger receptor for hemoglobin-haptoglobin, consistent with a role in red blood cell degradation and iron metabolism (Table S1). This led us to hypothesize that cluster 7 macrophages were not circulating cells as there were no CD206+CD169+CD163+ cells present in the blood of HSPC-engrafted MISTRG mice (Figure S6B).

To directly visualize human intravascular lung macrophages in MISTRG mice transplanted with human CD34+ HSPCs, lung sections were examined by electron microscopy. In addition to alveolar macrophages, we could detect cells with typical features of macrophages (irregular shape with pseudopods and “fuzzy” glycoskali coat) that were in close contact with endothelial cells in lung capillaries (Figures S6B and S6C). These intravascular macrophages contained phagosomes with red blood cells and electron-dense particles (likely siderosomes) and had a morphology distinct from alveolar macrophages. CD206+CD163+ macrophages were also present in human lung tissue sections, but it was difficult to distinguish by immunofluorescence microscopy whether they resided in the interstitial or intravascular space (Figure S6D).

To further prove that human lung macrophages labeled by intravenous CD45-PE antibody were located within the lung vasculature, we treated HSPC-engrafted MISTRG mice with clodronate by intravenous injection before intravascular labeling with anti-human CD45-PE antibody 24 h later (Figure 6C). Human CD206+CD169+ lung macrophages labeled with intravenous CD45-PE antibody were depleted, whereas extravascular macrophages were not reduced by pre-treatment with intravenous clodronate (Figure 6C). By days 3–7, the frequency of intravascular lung macrophages had returned to that of untreated mice (Figure 6C). There were no Ki67+ macrophages after clodronate depletion (Figure 6C), indicating that intravascular lung macrophages were restored by the differentiation from monocytes rather than by local proliferation.

Last, we investigated the cellular origin of human intravascular lung macrophages in MISTRG mice. Our scRNA-seq data showed a trajectory from CD14loCD16+ monocytes (cluster 3) toward C1Q macrophages (cluster 7), indicating that the two populations were related to each other (Figure 5C). This led us to hypothesize that intravascular lung macrophages originate from CD14loCD16+ blood monocytes. To test our hypothesis, we adoptively transferred dye-labeled CD14loCD16+ (as well as CD14+CD16+ and CD14+CD16+) blood monocytes into HSPC-engrafted MISTRG mice. Mice were treated with intravenous clodronate to deplete endogenous intravascular macrophages, i.e., to clear the intravascular niche, before monocyte transfer. These experiments revealed that all three monocyte subsets were able to give rise to intravascular lung macrophages (Figure 6D). Together with the developmental trajectory (Figure 5C), these results support the idea that intravascular lung macrophages develop from CD14loCD16+ monocytes that are derived from CD14+ monocytes.

Human intravascular lung macrophages have a distinctive gene signature

To gain further insights into their biology, we performed bulk RNA-seq of intravascular lung macrophages purified from HSPC-engrafted MISTRG mice (Figure 7A). Multi-dimensional scaling and differential gene expression showed that intravascular lung macrophages were distinct from intravascular monocytes and extravascular macrophages (Figures 7B and 7C; Figure S7A). Moreover, expression of C1QA, C1QB, and C1QC (and other cluster 7 and CD16+ monocyte-associated genes) was highest in intravascular lung macrophages (Figures 7D and S7B; Table S3), indicating that they corresponded to C1Q macrophages (cluster 7) as defined by scRNA-seq (Figure 5B). Apart from MAF and MAFB, intravascular lung macrophages were characterized by high expression of SPIC (Figures 7C and 7D), the master transcription factor for mouse red pulp macrophages (Kohyama et al., 2009). This suggested that intravascular lung macrophages are functionally related to red pulp macrophages. Accordingly, intravascular lung macrophages preferentially expressed genes involved in red blood cell...
turnover and iron metabolism (CD163, HMOX1, FTL, SLC40A1, SLC48A1) (Figure 7E). Finally, gene ontology analysis revealed an enrichment of genes related to leukocyte adhesion to the endothelium in intravascular macrophages compared to their extravascular counterparts (Figure S7C). In conclusion, we identify the transcriptional signature of human intravascular lung macrophages, which underlies their specific anatomical location and function.

**DISCUSSION**

As tissue-resident cells, macrophages play an important role in organ homeostasis and disease, yet little is known about the origin and ontogeny of human macrophages. Recent studies indicate great diversity of macrophages in the human lung (Bharat et al., 2016; Lambrechts et al., 2018; Lavin et al., 2017; Vieira Braga et al., 2019; Yu et al., 2016; Zilianis et al., 2019) and that circulating monocytes substantially contribute to the human lung macrophage compartment (Byrne et al., 2020). Here, we have mapped the monocytic origin of human lung macrophages in a humanized mouse model in vivo, defining their precursors and the developmental pathways for distinct types of human lung macrophages.

Our experiments identified CD14⁺CD16⁻ blood monocytes as the main precursors of HSPC-derived extravascular lung macrophages as well as of interstitial and alveolar macrophages, corresponding to equivalent populations in the human lung (Baharom et al., 2016; Bharat et al., 2016; Chakarov et al., 2019; Desch et al., 2016; Yu et al., 2016). The developmental trajectories from scRNA-seq suggested that human monocytes progressed through defined cellular stages before differentiating into distinct populations of mature lung macrophages. Our findings support the notion that HLA-DR⁺ monocytes, derived from CD14⁺CD16⁻ monocytes, actively migrate into the lung tissue and therefore represent a cellular intermediate in human lung macrophage differentiation from blood monocytes. Our data further revealed a transitional macrophage stage (cluster 6) with a gene signature indicating active protein synthesis and turnover. The transition from quiescence to cellular differentiation is dependent on increased ribosome biogenesis and protein synthesis (Sanchez et al., 2018) before ribosomal genes are suppressed during terminal differentiation of hematopoietic cells (Athanasiadis et al., 2017). Finally, we have shown that lung monocytes sequencially upregulate CD206 and CD169 during their maturation into interstitial and alveolar macrophages. This interpretation is consistent with human data showing that CD206⁺CD169⁺ macrophages develop shortly after birth (Alenghat and Esterly, 1984; Bharat et al., 2016), whereas CD206⁻CD169⁻ macrophages are present in stillborn infants (Bharat et al., 2016) and therefore develop earlier. Our findings on the migration of human monocytes into the lung and their differentiation into monocyte-derived macrophages are important for understanding the pathogenesis of lung diseases secondary to infection, chronic inflammation, and cancer. In particular, the identification of extravasating HLA-DR⁺ monocytes as precursors of human lung macrophages is relevant in acute lung injury in the context of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection, since circulating HLA-DR⁺ monocytes are depleted in severe coronavirus disease 2019 (COVID-19) (Arunachalam et al., 2020; Schulte-Schrepping et al., 2020).

We also uncovered a second pathway of human monocyte-to-lung macrophage differentiation. In contrast to CD14⁺CD16⁻ blood monocytes, CD14⁺CD16⁺ monocytes, which are dependent on M-CSF (Hofer et al., 2015), had low GM-CSF receptor expression, were confined to the lung vasculature, and preferentially differentiated into intravascular macrophages, in response to as yet unidentified signals from the endothelium. Therefore, our data indicate that the lung vasculature contains not only patrolling CD14⁺CD16⁻ and extravasating CD14⁺ monocytes but also intravascular macrophages. Despite populating the same anatomical niche, intravascular macrophages were distinct from blood monocytes in terms of morphology, phenotype, and gene expression.

Previous electron microscopy studies have described lung macrophages that are attached to the capillary endothelium. These pulmonary intravascular macrophages are constitutively present in species such as sheep and pigs, but not in rodents, at least in steady state (Brain et al., 1999; Longworth, 1997; Schneberger et al., 2012). Intravascular macrophages have also been described by electron microscopy in the human lung (Dehring and Wismar, 1989), but they remain poorly characterized. In this context, MISTRG mice could serve as a valuable in vivo model to study human intravascular macrophages.

The lung is a highly vascularized organ and therefore susceptible to not only airborne but also to blood-borne pathogens. Accordingly, human intravascular macrophages could act in the defense against bacteria that enter the lung vasculature. On the other hand, they could contribute to vascular injury by excessively aggregating in lung capillaries and producing pro-inflammatory factors in response to blood-borne microbes. Accordingly, it has been suggested that pulmonary intravascular macrophages may be involved in the pathogenesis of acute respiratory distress syndrome (Longworth, 1997). We discovered SPIC as a candidate...
transcription factor driving the development of human intravascular lung macrophages. Their transcriptional signature suggests that human intravascular lung macrophages are functionally related to other blood-filtering macrophages such as red pulp macrophages in the spleen and Kupffer cells in the liver. Furthermore, the gene signature of human intravascular lung macrophages fits well with a role in red blood cell turnover and iron recycling, thereby serving a homeostatic function in the lung.

Overall, our findings are consistent with the concept that, in addition to local tissue signals, the cellular origin and anatomical location of macrophage precursors dictates the functional specification of human macrophages and the spatial compartmentalization of tissue-resident immune responses. Given that macrophages have a central role in healthy lung function, our findings have implications for understanding the role of monocyte-derived macrophages in human lung homeostasis and disease.

LIMITATIONS OF STUDY

The MISTRG model does not recapitulate early stages of lung macrophage development in humans due to the absence of human fetal hematopoiesis and human macrophage precursors at birth. Moreover, human macrophages populating the mouse lung in MISTRG mice interact with the mouse epithelium and stroma. However, the mouse environment in MISTRG mice, expressing the critical human cytokines GM-CSF and M-CSF, seems sufficient to instruct human macrophage differentiation. In addition, some human immune cells that interact with lung macrophages, such as neutrophils, are not optimally represented in MISTRG mice. In MISTRG mice, HSPC-derived human blood monocytes populate an empty alveolar niche. Therefore, our findings are most relevant in the context of altered lung homeostasis, i.e., when resident macrophages are depleted due to lung injury and blood monocytes differentiate into monocyte-derived macrophages as it occurs for e.g., in SARS-CoV-2 infection (Bost et al., 2020; Liao et al., 2020). However, recent data highlight the importance of monocyte lung macrophage origin in healthy humans (Byrne et al., 2020), suggesting that our results are also relevant in non-pathological conditions. Finally, future studies are needed to define the abundance of intravascular macrophages in the healthy and diseased human lung.

STAR METHODS

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SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.immuni.2020.12.003.

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Figure 6. Human intravascular lung macrophages develop in HSPC-engrafted MISTRG mice

(A) Intravascular labeling of human CD45+ hematopoietic cells in MISTRG mice engrafted with CD34+ HSPCs by intravenous (IV) injection of anti-human CD45-PE antibody. Frequencies and phenotype of human CD206+CD169+ lung macrophages that are intravascular (IV hCD45-PE+) or in tissue (IV hCD45-PE–) are shown with IV clodronate 1, 3, or 7 days before intravascular labeling with IV human CD45-PE antibody. Data are represented as mean ± SEM.

(B) Electron microscopy of human intravascular lung macrophages in HSPC-engrafted MISTRG mice. Image shows a macrophage inside a lung capillary (cap) containing a phagocytosed red blood cell (RBC) (a) and another phagocytosed cell (b; white arrowheads). Zoomed image illustrates tight areas of contact between macrophage and endothelial cell (black arrowheads). Scale bar represents 5 μm.

(C) Frequencies of intravascular (IV hCD45-PE+) lung macrophages in control or clodronate-treated mice (n = 3–8). HSPC-engrafted MISTRG mice were injected with IV clodronate 1, 3, or 7 days before intravascular labeling with IV human CD45-PE antibody.

(D) IV adoptive transfer of dye-labeled human blood monocyte subsets into adult HSPC-engrafted MISTRG mice. Mice were treated with IV clodronate before monocyte transfer to deplete endogenous intravascular macrophages. Frequencies of dye+ human intravascular lung macrophages (IV hCD45-PE+CD11b+HLA-DR+CD206+) derived from injected monocytes are shown 3 weeks post-transfer (n = 3–8). Data are represented as mean ± SEM.

Data are representative of or combined from three experiments. See also Figure S6. (A), (C), and (D) was adapted from Servier Medical Art.
Figure 7. Human intravascular lung macrophages have a distinctive gene signature

(A) Bulk RNA-seq of purified intravascular (IV hCD45-PE-CD206-CD169-) monocytes, intravascular (IV hCD45-PE+CD206+CD169+) macrophages, and extravascular (IV hCD45-PE–CD206+CD169+) lung macrophages from HSPC-engrafted MISTRG mice.

(legend continued on next page)
AUTHOR CONTRIBUTIONS

E.E. designed, performed, and analyzed most experiments and wrote the paper. E.R. designed, performed, and analyzed experiments. K.P.T. analyzed scRNA-seq data. I.C.R. performed and analyzed some experiments. N.S., A.A., and Y.G. helped with mouse experiments. T.H. performed immunohistochemistry. C.S. helped prepare scRNA-seq libraries. D.S. performed immunofluorescence microscopy. N.M. and J.M. provided cells from human lung tissue. R.L. and A.S.-S. provided human BAL cells. J.B. supervised immunohistochemistry. M.C.I.K. supervised immunofluorescence microscopy and provided anti-human MARCO antibody. E.J.V. supervised scRNA-seq analysis. T.W. conceived and supervised the study, designed and analyzed experiments, and wrote the paper.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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Immunity 54, 259–275, February 9, 2021
### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| Mouse monoclonal anti-human CD3 Biotin | BioLegend | Cat#300404; RRID:AB_314058 |
| Mouse monoclonal anti-human CD11b BUV737 | BD Biosciences | Cat#564443; RRID:AB_2738811 |
| Rabbit monoclonal anti-human CD14 (Clone EPR3653) | Cell Marque | Cat#114R; RRID:AB_2827391 |
| Mouse monoclonal anti-human CD14 BV421 | BioLegend | Cat#301830; RRID:AB_10959324 |
| Mouse monoclonal anti-human CD16 FITC | BioLegend | Cat#302006; RRID:AB_314206 |
| Mouse monoclonal Biotin anti-human CD19 | BioLegend | Cat#302204; RRID:AB_314234 |
| Mouse monoclonal anti-human CD45 PE-Cy7 | BioLegend | Cat#304014; RRID:AB_314402 |
| Mouse monoclonal anti-human CD56 Biotin | BioLegend | Cat#318320; RRID:AB_893390 |
| Mouse monoclonal anti-human CD66abc Biotin | Miltenyi Biotec | Cat#130-119-848; RRID:AB_2751886 |
| Mouse monoclonal anti-human CD68 (clone PG-M1) | Dako (Agilent) | Cat # M0876; RRID:AB_2074844 |
| Mouse monoclonal anti-human CD116 PE (Clone 10D6) | BioLegend | Cat#305908; RRID:AB_2085686 |
| Mouse monoclonal anti-human CD163 BV650 (Clone EDH6-1) | BD Biosciences | Cat#563888; RRID:AB_2738468 |
| Rabbit polyclonal anti-human CD169 | Atlas Antibodies | Cat#HPA053457; RRID:AB_2682158 |
| Mouse monoclonal anti-human CD169 APC | BioLegend | Cat#346008; RRID:AB_11147948 |
| Rabbit polyclonal anti-human CD206 | Abcam | Cat#ab64693; RRID:AB_1523910 |
| Mouse monoclonal anti-human CD206 BV605 | BD Biosciences | Cat#740417; RRID:AB_2740147 |
| Mouse monoclonal anti-human CD206 AF488 | BioLegend | Cat#321114; RRID:AB_571875 |
| Mouse monoclonal anti-human HLA-DR BUV395 | BD Biosciences | Cat#564040; RRID:AB_2738558 |
| Mouse monoclonal anti-human Ki67 BV786 | BD Biosciences | Cat#563756; RRID:AB_2732007 |
| Mouse monoclonal anti-human MARCO | Mikael C I Karlsson Lab | https://doi.org/10.1016/j.celrep.2016.04.084 |
| Mouse monoclonal anti-human MARCO AF555 | Mikael C I Karlsson Lab | https://doi.org/10.1016/j.celrep.2016.04.084 |
| Mouse monoclonal anti-human NKP46 Biotin | BioLegend | Cat#331906; RRID:AB_1027671 |
| Rat monoclonal anti-mouse CD11b AF700 | BioLegend | Cat#101222; RRID:AB_493705 |
| Armenian hamster monoclonal anti-mouse CD11c PE-Cy7 | BioLegend | Cat#117318; RRID:AB_493568 |
| Rat monoclonal anti-mouse CD31 AF647 | BioLegend | Cat#102516; RRID:AB_2161029 |
| Rat monoclonal anti-mouse CD45 FITC | BioLegend | Cat#103108; RRID:AB_312973 |
| Rat monoclonal anti-mouse CD45 AF700 | BioLegend | Cat#103128; RRID:AB_493715 |
| Mouse monoclonal anti-mouse CD64 PE-Dazzle594 | BioLegend | Cat#139319; RRID:AB_2566558 |
| Rat monoclonal anti-mouse CD68 (clone FA-11) | Abcam | Cat#ab53444; RRID:AB_589007 |

(Continued on next page)
| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Rat monoclonal anti-mouse F4/80 APC | BioLegend | Cat#123116; RRID:AB_893481 |
| Rat monoclonal anti-mouse I-A/I-E BV785 | BioLegend | Cat#107645; RRID:AB_2565977 |
| Rat monoclonal anti-mouse Ly6C BV711 | BioLegend | Cat#128037; RRID:AB_2562630 |
| Rat monoclonal anti-mouse Ly6G APC-Cy7 | BioLegend | Cat#127624; RRID:AB_10640819 |
| Rat monoclonal anti-mouse SiglecF PE | BD Biosciences | Cat#552126; RRID:AB_394341 |
| Mouse monoclonal IgG1, κ Isotype Ctrl (FC) APC | BioLegend | Cat#400121; RRID:AB_326443 |
| Goat polyclonal anti-mouse IgG (H+L) AF555 | Thermo Fisher Scientific | Cat#A-21422; RRID:AB_2535844 |
| Goat polyclonal anti-mouse IgG (H+L) AF647 | Thermo Fisher Scientific | Cat#A-21235; RRID:AB_2535804 |

**Biological samples**

| Biological samples | Source | N/A |
|--------------------|--------|-----|
| Umbilical cord blood and Buffy coats | Karolinska University Hospital, Stockholm | N/A |
| Bronchoalveolar lavage (BAL) fluid | As previously described in Baharom et al. 2016 | N/A |
| Human lung cells | As previously described in Marquardt et al. 2019 | N/A |

**Chemicals, peptides, and recombinant proteins**

| Chemicals, peptides, and recombinant proteins | Source | N/A |
|-----------------------------------------------|--------|-----|
| Clodronate liposomes | Liposoma | Cat#C-005 |
| Collagenase IV | Sigma | Cat#C5138 |
| DNase I | Sigma | Cat#DN25 |
| Fixable Viability Dye eFluor 506 | Invitrogen | Cat#65-0866-14 |
| LPS (from E.coli O55:B5) | Sigma | Cat#L4524 |
| LymphoPrep | Fisher Scientific | Cat#11508545 |
| Microsphere Fluoresbrite® PC red/PE beads (0.5 μm) | Polysciences | Cat#19507-5 |
| pHrodo Red E. coli BioParticles | Thermo Fisher Scientific | Cat#P35361 |
| Poly(I:C) | Sigma | Cat#P9582 |
| Streptavidin Brilliant Violet 711 | BioLegend | Cat#405241 |
| Trizol | Life Technologies | Cat#15596-018 |

**Critical commercial assays**

| Critical commercial assays | Source | N/A |
|----------------------------|--------|-----|
| BCA protein assay kit | Fisher Scientific | Cat#10678484 |
| CD34+ Microbead kit | Miltenyi Biotec | Cat#130-046-702 |
| CellTrace Violet Cell Proliferation Kit | Thermo Fisher Scientific | Cat#C34557 |
| Chromium Single Cell 3’ Library & Gel Bead kit v2 | 10X Genomics | Cat#120237 |
| EasySep Human Monocyte Enrichment kit without CD16 depletion | StemCell Technologies, Inc. | Cat#19058 |
| Foxp3/Transcription Factor Staining Buffer Set | Thermo Fisher Scientific | Cat#00-5523-00 |
| Nextera XT DNA library preparation kit | Illumina | Cat#FC-131-1024 |
| PKH26 Red Fluorescent Cell Linker Kit for General Cell Membrane Labelling | Sigma | Cat#PKH26GL |
| SMART-Seq v4 Ultra Low Input RNA kit | Takara | Cat#634889 |
| SuperScript IV First-Strand Synthesis System | Life Technologies | Cat#18091050 |

(Continued on next page)
## Deposited data

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Single-cell RNA-sequencing data | Human lung myeloid cells from MISTRG mice (Day 21) | [https://doi.org/10.17044/scilifelab.13297784.v1](https://doi.org/10.17044/scilifelab.13297784.v1) |
| Single-cell RNA-sequencing data | Human lung myeloid cells from MISTRG mice (Day 36) | [https://doi.org/10.17044/scilifelab.13297784.v1](https://doi.org/10.17044/scilifelab.13297784.v1) |
| Bulk RNA-sequencing data | Human intravascular/extravascular lung monocytes & macrophages from MISTRG mice | [https://doi.org/10.17044/scilifelab.13297784.v1](https://doi.org/10.17044/scilifelab.13297784.v1) |

## Experimental models: organisms/strains

| Mouse: MISTRG | Rongvaux et al., 2014 | N/A |
| Mouse: MITRG | Rongvaux et al., 2014 | N/A |

## Oligonucleotides

| Primer for quantitative RT-PCR: Hprt Forward | Sigma | N/A |
| Primer for quantitative RT-PCR: Hprt Reverse | Sigma | N/A |
| Probe for quantitative RT-PCR: Hprt: [6FAM]TG TTGATAAGGAGGATGCAAGGCA | Sigma | N/A |

| TaqMan Assay: Human IL6 | Life Technologies | Hs00174131_m1 |
| TaqMan Assay: Mouse Il6 | Life Technologies | Mm00446690_m1 |
| TaqMan Assay: Human TNF | Life Technologies | Hs01113624_g1 |
| TaqMan Assay: Mouse Tnf | Life Technologies | Mm00443258_m1 |

## Software and algorithms

| Cell Ranger 3.0.1 | 10X Genomics | [https://support.10xgenomics.com/single-cell-gene-expression/software/pipelines/latest/what-is-cell-ranger](https://support.10xgenomics.com/single-cell-gene-expression/software/pipelines/latest/what-is-cell-ranger) |
| DESeq2 1.22.2 | Bioconductor | [https://bioconductor.org/packages/release/bioc/html/DESeq2.html](https://bioconductor.org/packages/release/bioc/html/DESeq2.html) |
| FastQC 0.11.8 | Babraham Bioinformatics | [https://www.bioinformatics.babraham.ac.uk/projects/fastqc/](https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) |
| FlowJo 10 | Tree Star | [https://www.flowjo.com/solutions/flowjo/downloads](https://www.flowjo.com/solutions/flowjo/downloads) |
| ggplot2 4.0.2 | R | [https://cran.r-project.org/web/packages/ggplot2/index.html](https://cran.r-project.org/web/packages/ggplot2/index.html) |
| GraphPad Prism 7 | GraphPad | [https://www.graphpad.com](https://www.graphpad.com) |
| ImageJ | Fiji | [https://imagej.net/Fiji](https://imagej.net/Fiji) |
| MASS | R 4.0.2 | [https://cran.r-project.org/web/packages/MASS/index.html](https://cran.r-project.org/web/packages/MASS/index.html) |
| NDP.view2 | Hamamatsu | [https://www.hamamatsu.com/us/en/product/type/U12388-01/index.html](https://www.hamamatsu.com/us/en/product/type/U12388-01/index.html) |
| Seurat 3.1.3 | Satija Lab | [https://satijalab.org/seurat/](https://satijalab.org/seurat/) |
| Slingshot 1.4.0 | Bioconductor | [https://bioconductor.org/packages/release/bioc/html/slingshot.html](https://bioconductor.org/packages/release/bioc/html/slingshot.html) |
| STAR 2.6.1d | GitHub | [https://github.com/alexdobin/STAR/releases](https://github.com/alexdobin/STAR/releases) |
| WebGestalt 2019 | Zhang Lab | [http://www.webgestalt.org](http://www.webgestalt.org) |
| Zen | Zeiss | [https://www.zeiss.com/microscopy/int/products/microscope-software/zen.html](https://www.zeiss.com/microscopy/int/products/microscope-software/zen.html) |
RESOURCES AVAILABILITY

Lead Contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact Tim Willinger (tim.willinger@ki.se).

Materials availability
Use of MISTRG mice requires Material Transfer Agreements with Regeneron Pharmaceuticals and Yale University.

Data and code availability
The scRNA-seq and bulk RNA-seq data derived from human lung myeloid cells in MISTRG mice have not been deposited in a public repository due to the European General Data Protection Regulation (GDPR) in order to protect human privacy, but are available from the corresponding author on request. The datasets will be deposited in a GDPR-compliant local European Genome-Phenome Archive (EGA)-node as soon as the service is available. Data access requests may be submitted to the corresponding author through the DOI link (https://doi.org/10.17044/scilifelab.13297784.v1) at the National Bioinformatics Infrastructure Sweden repository. Data access will be evaluated according to GDPR and Swedish legislation and any data that can be shared will be released via a data access agreement.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Mice
MISTRG mice homozygous for the human genes encoding M-CSF, IL-3, GM-CSF, SIRPa, and TPO in the Rag2−/− Il2rg−/− background were previously described (Rongvaux et al., 2014). For this study, we used an improved version of MISTRG mice with a genetically targeted SIRPA allele (Deng et al., 2015), instead of SIRPA transgene as in the original MISTRG mice. For transplantation with human CD34+ HSPCs (see below), MISTRG mice heterozygous for genetically targeted SIRPA were used. These mice were derived from breeding MISTRG mice (homozygous for SIRPA) with MITRG mice (lacking the targeted SIRPA allele) (Rongvaux et al., 2014). MISTRG mice were used under Material Transfer Agreements with Regeneron Pharmaceuticals and Yale University. MISTRG mice were re-derived by embryo transfer at Karolinska Institutet and maintained in individually ventilated cages under specific pathogen-free conditions without any prophylactic antibiotics. Mice (both males and females) were generally used at 5-12 weeks post-transplantation with human CD34+ HSPCs. All mouse experiments were performed in accordance with protocols approved by the Linköping Animal Experimentation Ethics Committee.

Human tissues
Umbilical cord blood and buffy coats were obtained from Caesarean sections and the Blood Bank at Karolinska University Hospital Huddinge, respectively. BAL fluid was collected from healthy donors undergoing bronchoscopy and used frozen, as previously described (Baharom et al., 2016). Frozen (non-malignant) lung cells obtained from patients undergoing lobectomy for suspected lung cancer were collected as part of a previous study (Marquardt et al., 2019). The collection of all human tissues was approved by local Ethical Review Boards at Karolinska Institutet and Umeå University. Informed consent was obtained from all tissue donors following verbal and written information and the investigations were conducted according to the Declaration of Helsinki.

METHOD DETAILS

Transplantation with human CD34+ HSPCs
CD34+ cells were isolated from pooled cord blood by density gradient centrifugation and positive immunomagnetic selection using a CD34+ microbead kit (Miltenyi Biotec). Newborn MISTRG mice (3-5 days old) were transplanted with 5x10⁴-1x10⁵ human CD34+ cells (usually > 90% purity) by intrahepatic injection as previously described (Rongvaux et al., 2014). HSPCs were pooled from several donors for transplantation. Mice did not receive any irradiation as pre-conditioning before transplantation. At 6 weeks post-transplantation, the frequency of human CD45+ cells in the blood was determined by flow cytometry. In general, only mice with ≥ 10% human CD45+ cell engraftment were used for subsequent experiments. In some experiments, MISTRG mice received PBS or TLR ligands (Sigma) intranasally at 2 weeks post-transplantation with human CD34+ cells: 0.5 mg/kg body weight LPS (from E.coli O55:B5) or 2 mg/kg body weight Poly(I:C).

Adoptive transfer of human monocytes
Blood monocyte subsets were isolated from buffy coats and first enriched by negative immunomagnetic selection using the EasySep Human Monocyte Enrichment kit without CD16 depletion (StemCell Technologies, Inc.). Enriched monocytes were then further purified by cell sorting into CD14+CD16-, CD14+CD16+, and CD14loCD16+ subsets (purity ≥ 95%). For the adoptive transfer experiments in Figure 3E, adult (8-11 weeks old) MISTRG mice, not previously engrafted with human CD34+ HSPCs, received two injections of 1x10⁶ purified CD14+CD16- or CD14+CD16+ monocytes via the tail vein with one day apart. For the adoptive transfer experiments in Figure 6D, adult (6-15 weeks old) MISTRG mice, engrafted with human CD34+ HSPCs, were used as recipients and pre-treated...
with 100 µL clodronate (Liposoma) by tail vein injection. 24 h later, recipient mice received two intravenous injections (2–4 days apart) of 2x10^6 purified CD14^-CD16^-, CD14^-CD16^+, or CD14^loCD16^- blood monocytes that were labeled with either 10 µM CellTrace Violet or 4 µM PKH26 before transfer. In both types of experiments, recipient mice were analyzed 3 weeks after monocyte transfer.

**Fate-mapping of human monocytes**

To fate map CD14^-CD16^- monocytes, MISTRG mice were injected intravenously with 100 µL of 0.5 µm-microsphere Flurosbrite® PC red/PE beads (Polysciences; diluted 1:10), 5 weeks after transplantation with human CD34^- cells. With this protocol, injected beads were first captured by CD14^-CD16^- blood monocytes that then differentiated into CD14^-CD16^- monocytes in the blood at the time of analysis. To fate map CD14^-CD16^- monocytes, MISTRG mice engrafted with CD34^- cells were first intravenously injected with 100 µL clodronate liposomes (Liposoma; 10 µL/g) to deplete blood monocytes. 24 h after clodronate administration, mice were intravenously injected with 100 µL microsphere beads. In this case, beads were taken up by recently generated CD14^-CD16^- monocytes released from the bone marrow that had not yet differentiated into CD14^-CD16^- monocytes by the time of analysis. Mice were bled 3 days after the bead injection to assess the efficiency and specificity of the monocyte labeling by flow cytometry. Mice were sacrificed 7 days after bead injection (i.e., 6 weeks post-transplantation with CD34^- cells), lungs harvested, and the frequency of fluorescent bead^+ lung macrophages determined by flow cytometry.

**Isolation of immune cells from MISTRG mice**

Lungs were perfused with 10 mL ice-cold PBS and digested in RPMI 1640/5% FCS with 0.2 mg/mL of collagenase IV (Sigma) and 0.02 mg/mL of DNase I (Sigma) for 60 min at 37° C. Digested cells were then passed sequentially through 18 and 20G needles before density gradient centrifugation using Lymphoprep (Fisher Scientific). BAL fluid was collected by inflating the lungs three times with 0.8 mL PBS via a catheter inserted into the trachea. BAL fluid was then centrifuged, the pellet resuspended in RPMI 1640/5% FCS, and BAL cells purified for flow cytometry by density gradient centrifugation. The supernatants were frozen at −80° C and the total protein concentration in BAL supernatants determined using the Bicinchoninic acid assay (BCA) kit (Thermo Fisher Scientific) according to the manufacturer’s instructions. Blood was taken by cardiac puncture and diluted in 200 units/mL heparin (Sigma). Erythrocytes were removed using red blood cell lysis buffer (obtained from Karolinska University Hospital) and the remaining immune cells stained for flow cytometry analysis.

**Intravascular cell labeling**

Engrafted MISTRG mice were injected intravenously with 2 µg of PE- or PE-Cy7-conjugated anti-human CD45 antibody (Biolegend or BD Biosciences, clone HI30) to label human hematopoietic cells in the blood and the lung vasculature. Mice were sacrificed 5 min after injection and lungs harvested without prior perfusion. Lung immune cells were isolated as above and stained with APC-Cy7^-conjugated anti-human CD45 antibody and other antibodies ex vivo for flow cytometry as described below. As a positive and negative control, cells from blood and BAL fluid were used, respectively. Only lung samples from mice with successful intravascular labeling (> 90% CD45^-PE/PE-Cy7^- in blood) were used for analysis.

**Flow cytometry and cell sorting**

Single-cell suspensions from lung, BAL, and blood of MISTRG mice were stained with fluorochrome- or biotin-labeled anti-human (or anti-mouse) antibodies (see Key resources table) in FACS buffer (PBS/2% FCS) for 30 min on ice, followed by secondary staining with streptavidin-Brilliant Violet 711 for 30 min on ice. For MARCO surface staining, cells were incubated with unconjugated anti-human MARCO antibody followed by staining with AF647-conjugated anti-mouse IgG secondary antibody (Thermo Fisher Scientific). After surface staining, cells were stained with fixable viability dye-eFluor506 (eBioscience) according to the manufacturer’s instructions. For intracellular Ki67 staining, cells were surface- and viability-stained before incubation in 200 µL Foxp3 Fixation/Permeabilization working solution (Foxp3/Transcription Factor Staining Buffer Set, Thermo Fisher Scientific) for 1 h at 4° C. After two washes with Permeabilization Buffer, cells were stained with anti-Ki67 antibody (BD Biosciences) for 30 min at 4° C, followed by a wash with Permeabilization buffer. Cells were fixed in PBS/2% PFA, acquired on a LSR II Fortessa flow cytometer (BD Biosciences), and data were analyzed with FlowJoV10 software. The indicated cell populations were sorted into RPMI/30% FCS medium using BD FACSAria III (BD Biosciences) or MA900 cell sorter (Sony Biotechnology). Cell purity after sorting was > 90%–95%.

**Immunohistochemistry**

For immunohistochemistry, lungs from HSPC-engrafted MISTRG mice were flushed through the right ventricle with 5 mL PBS followed by 5 mL 4% PFA before 1 mL 4% PFA was gently instilled via the trachea. For histology of non-engrafted MISTRG mice, lung were perfused with 10 mL PBS before harvest. In both cases, MISTRG lungs were fixed in 4% PFA for 24–48 h and stored in 70% ethanol. Dehydrated lungs were embedded in paraffin, cut into 4 µm sections, and incubated at 60° C for 45 min. After deparaffinization lung sections were rehydrated by successive washes in Xylene and graded ethanol. Automated immunohistochemistry was performed on an Autostainer 48 instrument (Dako) as previously described (Kampf et al., 2012). After pretreatment (PT linker at pH6; Dako), sections were stained with anti-human CD68 (clone PG-M1 1:100; Dako), CD206 (polyclonal 1:2300; Abcam), CD169 (pretreatment at pH9, polyclonal 1:250; Atlas Antibodies), CD163 (clone 10D6 1:100; Novocastra), and CD14 (clone EPR3653 ready to use; Cell Marque) antibodies or with anti-mouse CD68 (clone FA-11 1:200; Abcam) antibody. Then, the slides were developed using diaminobenzidine (Dako) aided by enzymes (MACH2 HRP-Mouse polymer or MACH2 HRP Rabbit polymer; Biocare Medical),
counterstained with Mayer’s hematoxylin (Histolab) and mounted. The stained slides were scanned at 40x magnification and analyzed with the NDP.view2 software (Hamamatsu). At the start of the project specific staining patterns were confirmed for each marker on normal human lung tissue and on lung tissue from MISTRG mice that were not engrafted with human HSPCs.

**Immunofluorescence microscopy**

Lungs were fixed in 4% PFA, dehydrated in a 10% → 20% → 30% sucrose gradient, and frozen in OCT medium. 7 μm sections were cut and slides fixed in ice-cold acetone (Sigma) for 5 min. Prior to staining, sections were blocked with 5% BSA in PBS for 30 min at room temperature. Sections from MISTRG mice were stained with AF488-conjugated anti-human CD206 (Biolegend, clone 15-2) and AF555-conjugated MARCO (produced in-house) antibodies, as well as with AF647-conjugated anti-mouse CD31 (Biolegend, clone MEC13.3.) antibody for 30–60 min in PBS at room temperature in a humidified chamber before co-staining with the nuclear dye Hoechst (Thermo Fisher Scientific, dilution 1:5000) for 10 min. Sections from human lung tissue were stained with AF488-conjugated anti-human CD206 (Biolegend, clone 15-2) and unconjugated mouse anti-human CD163 (BIO-RAD, clone EDHu-1) antibodies. Sections were then stained with AF555-conjugated anti-mouse secondary antibody (Thermo Fisher Scientific) and Hoechst. Following staining slides were incubated with an autofluorescence quencher for 30 s (TrueBlack Lipofuscin, Biotium). Slides were mounted in ProLong Diamond Antifade Mountant (Thermo Fisher Scientific). Images were acquired on a Zeiss LSM800 confocal microscope, processed with Zen software, and analyzed in ImageJ (Fiji).

**Electron microscopy**

Perfusion-fixation was performed by flushing lungs through the right ventricle with 5 mL PBS followed by 5 mL of fixative (mixture of 3% glutaraldehyde and 1% formaldehyde in 0.1 M cacodylate supplemented with 0.1 M sucrose and 3 mM CaCl2, pH 7.4). Then, 1ml fixative was gently instilled via the trachea. Finally, the bilateral lung block was cut out, submerged in fixative, and stored at 4°C. Fixation was performed at room temperature in a humidified chamber before co-staining with the nuclear dye Hoechst (Thermo Fisher Scientific, dilution 1:5000) for 10 min. Sections from human lung tissue were stained with AF488-conjugated anti-human CD206 (Biolegend, clone 15-2) and unconjugated mouse anti-human CD163 (BIO-RAD, clone EDHu-1) antibodies. Sections were then stained with AF555-conjugated anti-mouse secondary antibody (Thermo Fisher Scientific) and Hoechst. Following staining slides were incubated with an autofluorescence quencher for 30 s (TrueBlack Lipofuscin, Biotium). Slides were mounted in ProLong Diamond Antifade Mountant (Thermo Fisher Scientific). Images were acquired on a Zeiss LSM800 confocal microscope, processed with Zen software, and analyzed in ImageJ (Fiji).

**scRNA-seq of human lung myeloid cells**

Live human CD45+/HLA-DR+/CD11b- mouse CD45- myeloid cells were sorted from the lungs of four different MISTRG mice, engrafted with the same pooled batch of human CD34+ cells, 36 days after transplantation. In a separate experiment, cells were isolated from three different MISTRG mice 21 days post-transplantation with the same pooled batch of human CD34+ cells. Single-cell libraries were prepared using the 10x Genomics Single Cell 3’ Library v2 kit according to the manufacturer’s instructions. Libraries were sequenced on a HiSeq-PE150 or Nextseq 550 (Illumina) and mapped to the human genome (GRCh37) using the Cell Ranger 3.0.1 pipeline (10x Genomics). We then used the R software package Seurat 3.1.3 for data pre-processing and quality control. Low-quality cells and doublets were filtered out based on having (i) < 200 and > 2500 unique genes and (ii) > 20% mitochondrial content. After removing low-quality cells and doublets, a total of 28,207 cells (day 36) and 2,514 cells (day 21) were retained for downstream analysis with Seurat. For data normalization, the “LogNormalize” method was used and 2,000 highly variable genes were identified per dataset. Unwanted sources of variation, such as mitochondrial contamination, were also removed during the normalization procedure. All samples were then integrated using the built-in anchor-based approach in Seurat 3.1.3. In the integrated datasets, 7,972 and 17,413 unique genes were detected for day 36 and day 21, respectively. Next, as a standard pre-processing step prior to linear dimensional reduction, data were scaled using linear transformation. Then, PCA was performed on the scaled data and the top 20 PCA components for cell clustering were determined based on the JackStraw procedure. A graph-based clustering approach was employed to identify cell clusters. For optimal cluster resolution, the FindClusters function in Seurat was set to 0.5. To visualize the clusters, non-linear dimensional reduction with UMAP was performed by taking as input the same 20 PCA components above. Differentially expressed genes in each cluster (cluster markers) were identified by using Wilcoxon rank sum test, with logfc, Threshold, and min.pct parameters equal or greater than 0.25 respectively. To visualize marker expression, in-built Seurat plotting functions DoHeatmap and FeaturePlot were used. The cell type identity was assigned to clusters based on the expression of known cell type-specific marker genes. In the day 36 analysis, we classified cluster 9 as neurons and cluster 8 as T lymphocytes based on their expression of synaptic (SYNGR1, CADM1) and T cell genes (CD3D, GNL1), respectively (Table S1). We attributed these minor contaminations to possible close interactions between lung macrophages and T cells or neurons, as described recently in mice (Chakarov et al., 2019; Ural et al., 2020).

**RNA-seq of intravascular lung macrophages**

Three populations of human lung myeloid cells (live human CD45+/HLA-DR+/CD11b- mouse CD45- cells) were sorted from HSPC-engrafted MISTRG mice (8-12 weeks old) after intravascular labeling with anti-human CD45-PE antibody: (1) Intravascular monocytes

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(IV CD45-PE*CD206*CD169*); (2) Intravascular macrophages (IV CD45-PE*CD206*CD169*); (3) Extravascular macrophages (IV CD45-PE*CD206*CD169*). For each of the four samples (i.e., four independent sorts), lung cells from 3-4 individual mice were pooled for sorting. Sorted cells (intravascular monocytes: ~20,000-40,000 per sample; intravascular macrophages: ~3,000-4,500 per sample; extravascular macrophages: ~6,000-12,000 per sample) were collected and frozen in RNaseasy Lysis Buffer (QIAGEN), total RNA extracted using RNaseasy Micro kit (QIAGEN), and RNA quality assessed with an Agilent Bioanalyser. cDNA libraries were prepared using 1 ng of total RNA with the SMART-Seq v4 Ultra Low Input RNA kit (Takara) and Nextera XT DNA library preparation kit (Illumina). Libraries were sequenced on an Illumina Nextseq 550 using using a V 2.75 cycle kit SR mode (Read 1: 75 cycles. Dual Index. Index 1: 8 cycles Index 2: 8 cycles). Reads were demultiplexed using bcl2fastq (v2.20.0) and quality assessed using FastQC (v0.11.8). Raw reads were aligned to the human genome (GRCh38) using STAR (v2.6.1d), followed by assignment of reads to genes using featureCounts (v1.5.1) Due to a low alignment rate (< 50%), one intravascular monocyte replicate was excluded from further analysis. Bioconductor package DESeq2 (v1.22.2) was used for count normalization and to identify differentially expressed genes between monocyte and macrophage populations, generating log2 fold changes, Wald test p-values, and p-values adjusted for multiple testing (Benjamini-Hochberg method). Genes with an adjusted p-value < 0.05 and log2 fold change > 1 were considered as differentially expressed between paired cell populations. Multidimensional scaling (MDS) and volcano plots were created in R (v4.0.2) using packages MASS and ggplot2. Gene Ontology over-representation analysis (Biological Process) was performed with WebGestalt (http://www.webgestalt.org) using default parameters.

Macrophage function in vivo
To assess cytokine induction, HSPC-engrafted MISTRG mice were treated intranasally with either PBS or TLR ligand (10 μg LPS from E.coli O55:B5 (Sigma)). Lungs were harvested 2 h after treatment for RNA extraction with TRIzol reagent (Invitrogen). After cDNA synthesis with the SuperScript First-Strand Synthesis System (Invitrogen), quantitative RT-PCR was performed on a P CRQuant Studio 5 Real-Time PCR system (Applied Biosystems) with primer-probe sets from Applied Biosystems (TNF, IL6, Tnf, Il6) or Sigma (Hprt). To assess phagocytic activity, HSPC-engrafted MISTRG mice received 3x10⁸ pHrodo Red E. coli BioParticles (or PBS as control) via the intranasal route. Lungs were harvested 3 h later for flow cytometric analysis.

QUANTIFICATION AND STATISTICAL ANALYSIS
Statistical parameters including number of biological replicates and repeat experiments, data dispersion and precision measures (mean and standard error of the mean (SEM)), and P values for statistical significance (α = 0.05) are reported in Figures and Figure Legends. Student’s t test was used to determine statistical significance between two groups. For multigroup comparisons, we applied one-way ANOVA with post hoc testing using Tukey’s Multiple Comparison Test. Statistical analysis was performed using GraphPad Prism 7.