© 2016. Published by The Company of Biologists Ltd | Development (2016) 143, 1318-1327 doi:10.1242/dev.129122

RESEARCH ARTICLE

Developmental origin of lung macrophage diversity

Serena Y. S. Tan and Mark A. Krasnow*

ABSTRACT

Macrophages are specialized phagocytic cells, present in all tissues, which engulf and digest pathogens, infected and dying cells, and debris, and can recruit and regulate other immune cells and the inflammatory response and aid in tissue repair. Macrophage subpopulations play distinct roles in these processes and in disease, and are typically recognized by differences in marker expression, immune function, or tissue of residency. Although macrophage subpopulations in the brain have been found to have distinct developmental origins, the extent to which development contributes to macrophage diversity between tissues and within tissues is not well understood. Here, we investigate the development and maintenance of mouse lung macrophages by marker expression patterns, genetic lineage tracing and parabiosis. We show that macrophages populate the lung in three developmental waves, each giving rise to a distinct lineage. These lineages express different markers, reside in different locations, renew in different ways, and show little or no interconversion. Thus, development contributes significantly to lung macrophage diversity and targets each lineage to a different anatomical domain.

KEY WORDS: Lineage tracing, Lung development, Lung macrophage, Parabiosis, Phagocytosis

INTRODUCTION

Macrophages are professional phagocytes found in all tissues. They are best known for their general functions in clearance of potential pathogens and dead cells, as responders to injury and mediators of inflammation and tissue repair, and for their roles in antigen presentation. However, macrophages in different tissues also have tissue-specific functions and structures, express different combinations of macrophage markers and show differences in their dependence on specific growth factors and responses to cytokines. For example, splenic red pulp macrophages remove senescent red blood cells and salvage iron (den Haan and Kraal, 2012), whereas brain microglia promote neuronal survival (Aguzzi et al., 2013) and lung macrophages catabolize surfactant (den Haan and Kraal, 2012; Shibata et al., 2001).

Historically, such tissue-specific specializations of macrophages were assumed to be conferred by tissue- and organ-specific microenvironments because all macrophages were thought to arise from a common source: circulating monocytes originating in the bone marrow (Aguzzi et al., 2013; van Furth and Cohn, 1968). However, the fetal yolk sac was subsequently identified as another source of macrophages, active before definitive hematopoiesis begins in the fetal liver (Takahashi et al., 1989), and these very early macrophage progenitors were proposed to colonize specific organs in the embryo and mature into self-renewing tissue macrophages, including the 'microglia' of the adult brain and 'cardiac macrophages' in the heart (Higashi et al., 1992; Lichanska and Hume, 2000; Robinson, 1984; Takeya and Takahashi, 1992). This idea was substantiated by modern genetic lineage-tracing experiments in mice, which confirmed the existence of self-renewing, embryonic-derived macrophages in the brain and heart, as well as other organs (Ginhoux et al., 2010), whereas in chick all yolk sac-derived macrophages may be lost after hatching (Garceau et al., 2015). A third source of tissue macrophages, the fetal liver, was also identified recently by adoptive transfer of fetal monocytes and shown to give rise to Langerhans cells, resident macrophages of the skin (Hoeffel et al., 2012). Thus, differences in developmental origin, as well as tissue-specific microenvironment, could contribute to the tissue-specific specializations of macrophages.

Recent studies show that macrophages within a tissue can also arise from different sources. For example, there appear to be three sources of adult skin macrophages (Langerhans cells): the fetal liver noted above, but also yolk sac and adult bone marrow (Hoeffel et al., 2012). Similarly, developmentally distinct subsets of cardiac macrophages have been identified (Epelman et al., 2014; den Haan and Kraal, 2012). However, in each of these cases the different sources appear to give rise to the same or interconvertible resident macrophage cell types (Aguzzi et al., 2013; Hoeffel et al., 2012; Epelman et al., 2014; den Haan and Kraal, 2012; Shibata et al., 2001). This suggests that tissue microenvironments are distinct and dominant over developmental origin in determining the specific structure and function of the resident macrophages. Our systematic study of lung macrophage development shows just the opposite, providing evidence for a dominant role of development in dictating the major classes of macrophages within a tissue.

Two major classes of lung macrophages are recognized. The most abundant and best-studied population is alveolar macrophages, which reside within the lumen of the alveolus, directly exposed to air and the environment but closely apposed to the alveolar epithelium. Besides phagocytosis of foreign particles, alveolar macrophages are essential for catabolizing the surfactant that lines the surface of the alveoli and helps prevent their collapse (Aguzzi et al., 2013; Shibata et al., 2001; van Furth and Cohn, 1968). The other major population is interstitial macrophages, which comprise 30-40% of lung macrophages (Bedoret et al., 2009; den Haan and Kraal, 2012; Lehnert, 1992; Takahashi et al., 1989). Only the thin alveolar wall separates alveolar from interstitial macrophages, which occupy the same narrow interalveolar space as alveolar capillaries, fibroblasts and other mesenchymal cells. Interstitial macrophages are thought to have roles in tissue remodeling and maintenance as well as antigen presentation (Aguzzi et al., 2013; Higashi et al., 1992; Lichanska and Hume, 2000; Robinson, 1984; Schneberger et al., 2011; Takeya and Takahashi, 1992), and influencing dendritic cell functions to prevent airway allergy

Department of Biochemistry and HHMI, Stanford University School of Medicine, Stanford, CA 94305-5307, USA.

*Author for correspondence (krasnow@stanford.edu)

Received 5 August 2015; Accepted 21 February 2016

Development (2016) 143, 1318-1327 doi:10.1242/dev.129122

(Bedoret et al., 2009; Ginhoux et al., 2010; den Haan and Kraal, 2012; Shibata et al., 2001).

Histological studies suggested that embryonic lung macrophages derive from the yolk sac (Aguzzi et al., 2013; Hoeffel et al., 2012; Sorokin et al., 1984, 1992; van Furth and Cohn, 1968), and subsequent immunohistochemical studies suggested that these fetal macrophages differentiated into interstitial macrophages, then to alveolar macrophages in the postnatal lung (Higashi et al., 1992; Hoeffel et al., 2012; Takahashi et al., 1989), which were then maintained by circulating monocytes with interstitial macrophages as putative intermediates. The role of interstitial macrophages as developmental intermediates was supported by observations that proliferation or replenishment of lung macrophages in the interstitial compartment often preceded that of the alveolar compartment following injury, depletion or explant culture (Bowden and Adamson, 1972; Bowden et al., 1969; Higashi et al., 1992; Landsman and Jung, 2007; Lichanska and Hume, 2000; Robinson, 1984; Takeya and Takahashi, 1992), and by ultrastructural and immunohistochemical studies suggesting that interstitial macrophages were intermediate in size and molecular phenotype between monocytes and alveolar macrophages (Cai et al., 2014; Ginhoux et al., 2010; Sebring and Lehnert, 1992). These early models suggested two different sources of lung macrophages (fetal yolk sac and adult bone marrow), but as for skin and cardiac macrophages, developmental origin is unimportant in the models because both sources were thought to give rise to interstitial and alveolar macrophages, with interstitial macrophages as obligate progenitors of alveolar macrophages. However, early thymidine-labeling studies and recent parabiosis experiments provide evidence that alveolar macrophages can divide, self-renew, and maintain themselves independently of the circulation (Golde et al., 1974; Hoeffel et al., 2012; Murphy et al., 2008; Epelman et al., 2014; Hashimoto et al., 2013), and recent adoptive transfer and lineage tracing of fetal monocytes suggest that alveolar macrophages derive from fetal liver, not yolk sac (Guilliams et al., 2013; Hoeffel et al., 2012), implying a separate developmental origin from interstitial macrophages.

To address the questions of when and how each lung macrophage subpopulation arises during development, how are they maintained, and whether they interconvert, we carried out a systematic study of lung macrophage development in mice. We used immunostaining to localize macrophage subpopulations in the developing and mature lung, along with genetic lineage tracing and parabiosis experiments to characterize the development and maintenance of lung macrophages throughout the mouse life span, from early embryo to adult. We show that there are three distinct lineages of lung macrophages that arrive at different times, reside in different locations, renew in different ways, and show little or no interconversion.

RESULTS

Macrophage markers reveal two intermingled macrophage populations in the developing mouse lung

To molecularly characterize developing lung macrophages and visualize their distribution in the lung, we stained sections of mouse lungs at embryonic day (E) 15.5 for ten macrophage markers (Table S1). We used lungs from the *c-fms-GFP* ('MacGreen') transgenic line (Epelman et al., 2014; Rae et al., 2007) in which all macrophages express cytoplasmic GFP. The microsialin CD68 was expressed by all c-fms$^+$ (also known as Csf1r) macrophages (Fig. S1). Two other macrophage markers, cell surface glycoprotein F4/80 (also known as Emr1 and Adgre1) and galactose-specific lectin Mac2 (Lgals3), distinguished two large subpopulations, one that expressed high F4/80 and no Mac2, and another that expressed Mac2 and low or no detectable F4/80 (Fig. 1A,B; Table S1). There was no overlap between the two subpopulations (Fig. 1D), and the two subpopulations accounted for all c-fms$^+$ lung macrophages (Fig. 1C). Macrophages expressing high F4/80 ('F4/80 macrophages') were variable in shape. Some were ellipsoid, though most (~60%) had a distinctive irregular morphology with

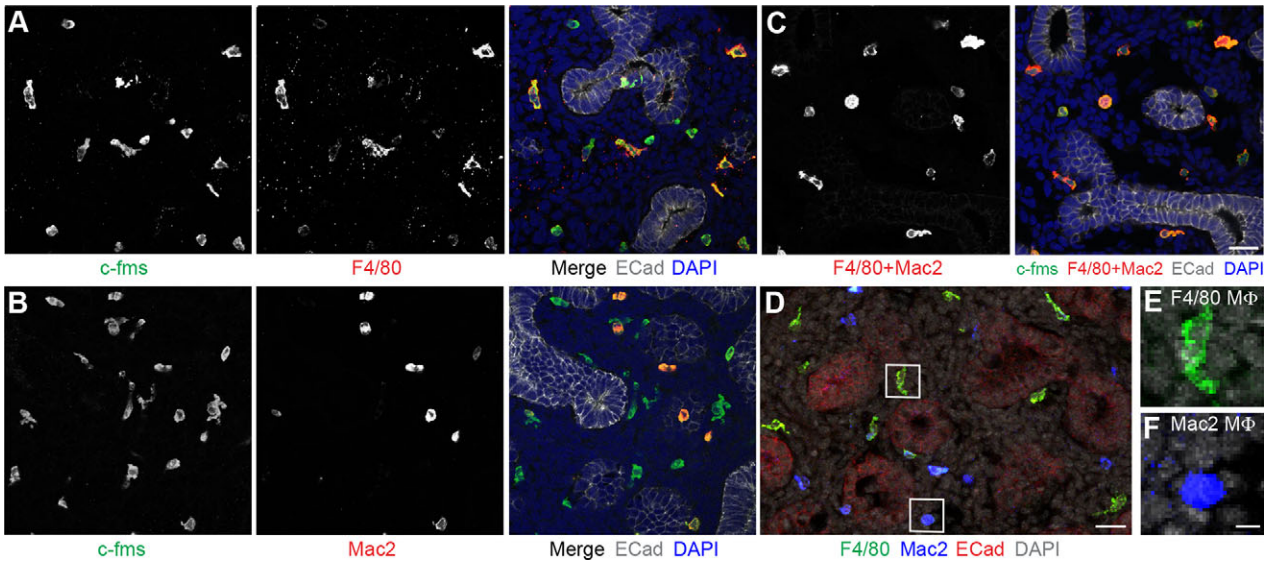

Fig. 1. Marker expression and morphology of mid-embryonic lung macrophages. (A-C) Lung sections from E15.5 c-fms-EGFP mice immunostained as indicated for macrophage markers F4/80 and Mac2, and for E-cadherin (gray) to show airway epithelium. All lung macrophages express c-fms-EGFP (green) and major subsets express F4/80 (A) or Mac2 (B). Co-staining for F4/80 and Mac2 (C) show that all c-fms$^+$ macrophages express either high F4/80 or Mac2. (D) E15.5 wild-type (C57BL/6J) lung co-stained for F4/80 and Mac2. F4/80 and Mac2 label complementary macrophage subsets. (E,F) Close-ups of the boxed regions in D. F4/80$^+$ macrophages are more irregularly shaped with pseudopodia (E), whereas Mac2$^+$ macrophages are more regular and elliptical (F). DAPI, nuclear stain. Scale bars: 20 µm (A-D), 5 µm (E,F).

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pseudopods (Fig. 1E). Macrophages expressing Mac2 (‘Mac2 macrophages’) were more uniform and rounded in appearance (Fig. 1F). F4/80 and Mac2 macrophages were intermingled throughout the lung interstitium (Fig. 1D); none was detected within the airway lumen.

Dynamic changes in macrophage subpopulations during embryonic development

Examination of the F4/80 and Mac2 macrophage subpopulations during embryonic development showed that F4/80 and Mac2 macrophages appeared in the developing lung with different kinetics (Fig. 2). F4/80 macrophages were detected early (Fig. 2A), beginning at E10.5 (Fig. 2E) when the primordial lung buds from the foregut; no Mac2 macrophages were detected at this time. The number of lung macrophages increased substantially over the next several days (Fig. 2B,E), dominated by F4/80 macrophages. The first Mac2 macrophages were detected at E12.5 (Fig. 2E). The Mac2 subpopulation increased slowly at first, but between E15.5 and E16.5, there was a dramatic increase in Mac2 macrophages, and they overtook the F4/80 macrophages at E15.5 and remained the dominant subpopulation throughout the rest of embryonic development (Fig. 2C-E). Around E16, some Mac2 macrophages also began to express detectable levels of F4/80, but it never reached the levels expressed by F4/80 macrophages (Table S1). The distinct morphologies of F4/80 and Mac2 macrophages (Fig. 1E,F) were maintained throughout embryonic development, and the two subpopulations remained intermingled throughout the interstitium during this time, with few detected in the airway lumen. Thus, there are two major phases of embryonic lung macrophage development: an early phase (E10-E15) dominated by F4/80 macrophages, and a late phase (E16-birth) dominated by Mac2 macrophages.

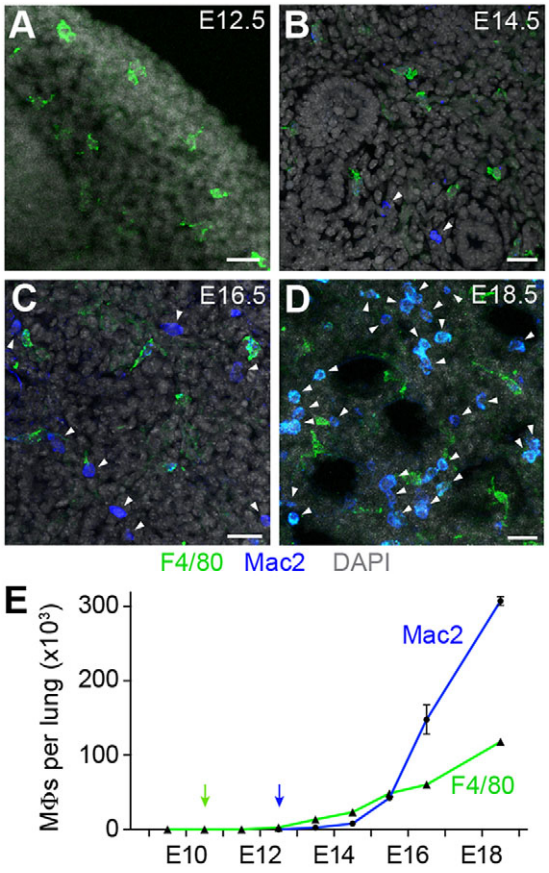

Fig. 2. Dynamics of F4/80 and Mac2 macrophage subpopulations in the developing lung. (A-D) Sections through wild-type lungs of the indicated embryonic ages immunostained to show F4/80^{+} (green) and Mac2^{+} (blue, arrowheads) macrophages. The two subpopulations and their kinetics are consistent with the F4/80-high and F4/80-low macrophage subpopulations previously described in the embryo (Schulz et al., 2012), and indeed low levels of F4/80 can be detected in some Mac2 macrophages especially after E15.5 (Table S1). Scale bars: 20 µm. (E) Quantification of abundance of F4/80^{+} and Mac2^{+} macrophages throughout embryonic lung development. F4/80 macrophages dominate early in lung development and Mac2 macrophages dominate after E15.5. Values shown are mean±s.e.m. for *n*=750-3570 macrophages scored in two or three lungs per time point. Values at early time points obscured by the *x*-axis are: E9.5 (0 per lung, F4/80 macrophages; 0 per lung, Mac2 macrophages), E10.5 (4±1; 0), E11.5 (39±5; 0), E12.5 (2600±81; 67±21). Arrows indicate the earliest developmental ages that F4/80 (green arrow) and Mac2 macrophages (blue arrow) were detected in the lung. Note that most of the error bars are very small and obscured by the data point symbols. Mϕs, macrophages.

Runx1 lineage tracing shows that F4/80 macrophages arise from yolk sac hematopoietic cells

To investigate the developmental origin and relationship of F4/80 and Mac2 macrophages, we performed a lineage-tracing study using mice expressing the tamoxifen-inducible *MER-Cre-MER* recombinase gene under control of the *Runx1* promoter. Runx1 is expressed in primitive hematopoietic cells, which are located exclusively in the extra-embryonic yolk sac between E7 and E8 (Samokhvalov et al., 2007). To label descendants of these early primitive hematopoietic cells, we induced recombination in pregnant *Runx1-MER-Cre-MER* (*Runx1*$^{Cre/wt}$); *Rosa26*$^{TdTomato/TdTomato}$ Cre-reporter mice using a single injection of tamoxifen at ∼E6.75, then examined embryonic lungs for Runx1 lineage-labeled cells 8 and 12 days later at mid (E14.5) and late (E18.5) embryogenesis. At both time points, 30-40% of the F4/80^{+} macrophages were labeled with the Runx1 lineage tag (Fig. 3A,B,D), matching the proportion (up to 40%) of microglia, a canonical yolk sac lineage, labeled by the Runx1 lineage tag (Ginhoux et al., 2010). By contrast, few (<5%) Mac2^{+} macrophages were labeled (Fig. 3A,C,D). This low-level labeling of Mac2 macrophages might result from labeling of hematopoietic precursors at the beginning of a subsequent wave of

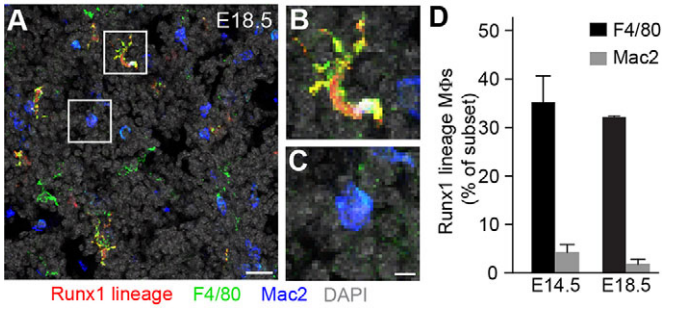

Fig. 3. Lineage tracing of yolk sac precursors in embryonic lung. (A-D) Section through E18.5 lung of a Runx1-CreER>TdTomato mouse (lineage trace in red), induced with tamoxifen at E6.75 to label yolk sac precursors, and immunostained to show F4/80 (green) and Mac2 (blue) macrophages. Lineage label marks F4/80 macrophages (A,B; yellow cells) but few Mac2 macrophages (A,C; blue cells). At this age, many Mac2 macrophages express low, variable levels of F4/80. Scale bars: 20 µm (A), 5 µm (B,C). (D) Quantification showing fraction of each macrophage subpopulation that expresses the Runx1 lineage label at E14.5 and E18.5. Values shown are mean+s.e.m.; *n*=210-1390 macrophages scored of each type in two lungs per time point. Mϕs, macrophages.

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macrophage development, because induction of the Runx1 lineage trace about a day later (E8) resulted in substantial labeling of Mac2 macrophages (data not shown). We conclude that many, and perhaps all, F4/80 macrophages derive from early primitive hematopoiesis in the yolk sac and persist through late embryogenesis, and few, if any, become Mac2 macrophages during this time. Thus, F4/80 and Mac2 macrophages appear to be largely or completely separate macrophage lineages in development. However, methods for specific and complete labeling of each subpopulation will be necessary to exclude the possibility of any interconversion between the lineages.

Some yolk sac-derived F4/80 macrophages persist postnatally at special locations

To investigate the fate of the early yolk sac-derived embryonic F4/80 macrophages after birth, we examined lungs of juvenile [postnatal day (P) 7] and adult (P60-P70) Runx1 lineage trace mice induced with tamoxifen at E6.75 as above. A small population of Runx1 lineage-labeled cells remained in the adult lung, and nearly all (>95%) were interstitial macrophages. However, they were not distributed homogeneously throughout the interstitium (Fig. 4), as they were during embryogenesis (Fig. 3). Instead, the Runx1 lineage-labeled macrophages localized with a peripheral bias, along the mesothelium, as well as perivascularly, along large vessels, with very few remaining in the central lung parenchyma (Fig. 4A,B). Cell counts showed that between late embryonic and adult life there was a 200-fold increase in the peripheral density of Runx1 lineage-labeled macrophages relative to their central density

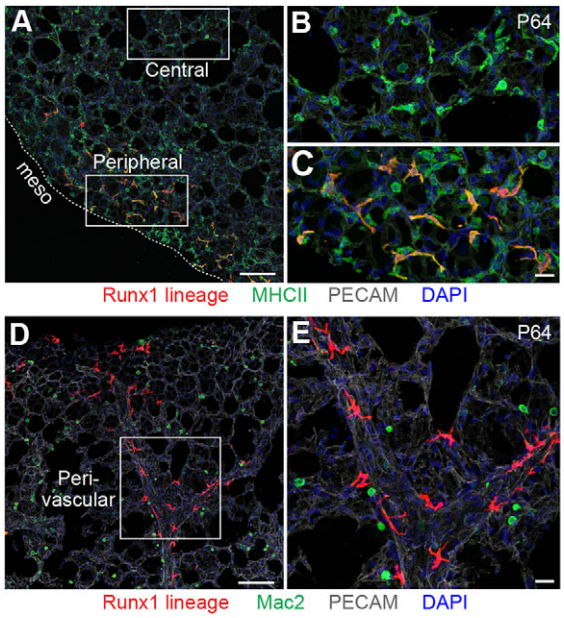

Fig. 4. Lineage trace of yolk sac precursors in the adult lung. (A-E) Adult (P64) lungs from Runx1-CreER>TdTomato lineage trace induced at E6.75 as in Fig. 3 and immunostained for MHCII (green; A-C) or Mac2 (green; D,E) and PECAM (blood vessels, gray) and counterstained with DAPI (blue). Note that lineage-labeled interstitial macrophages express MHCII macrophage marker (yellow cells in A and close-up of peripheral region shown in C) but not Mac2 macrophage marker (red cells in D and perivascular close-up in E) and localize to the periphery along mesothelium (meso; A,C) and large blood vessels (D,E). The macrophages that are not lineage labeled are MHCII^{+} interstitial macrophages (green cells in A and central close-up in B), located in the more central parenchyma further from the mesothelium, and Mac2^{+} alveolar macrophages (green cells in D,E). Scale bars: 100 µm (A,D), 20 µm (C,E).

(see Materials and Methods). This peripheral and perivascular concentration of Runx1 lineage-labeled macrophages was also observed in juvenile (P7) lungs (Fig. S3), implying that the transition from a broad distribution to this restricted interstitial distribution of yolk sac-derived macrophages occurs early in postnatal life. The change could be due either to migration of the yolk sac-derived F4/80 macrophages towards the mesothelium and blood vessels, and/or to their preferential survival or proliferation at these locations coupled with replacement of the central parenchymal macrophages by postnatal influx of a new macrophage population (see below).

Postnatal gene expression changes in the parenchymal F4/80 macrophage population

The disappearance of yolk sac-derived macrophages from the lung parenchyma during postnatal life suggested replacement by a new population. To better understand the postnatal dynamics of the parenchymal interstitial macrophage population, we investigated their marker expression pattern and how it changes during postnatal life. Lung macrophages in postnatal and adult c-fms-EGFP mice were immunostained for nine macrophage markers (Table S1); CD68 was the only one that colocalized with all EGFP^{+} macrophages (Fig. S1). By contrast, expression of the F4/80 and MHCII (histocompatability 2, H2) macrophage markers in the interstitial lung macrophage population showed dynamic and reciprocal changes during postnatal development. In the first postnatal week, F4/80 was expressed on almost all Mac2-negative interstitial macrophages (Fig. 5A,E). However, by the end of the third postnatal week through adulthood, few if any interstitial macrophages expressed F4/80 (Fig. 5B,E). Conversely, few lung macrophages expressed MHCII in the first postnatal week (Fig. 5C,E), but nearly all interstitial macrophages expressed MHCII by the end of the third postnatal week (Fig. 5D,E).

A second population of F4/80 interstitial macrophage arrives early in postnatal life

The observed changes in marker expression in the interstitial macrophage population during early postnatal life could result from a change in gene expression of the resident macrophages or the arrival of a new macrophage population. To distinguish between these possibilities, we characterized expression of an F4/80 lineage label in *F4/80$^{Cre/+}$;Rosa26^{TdTomato}* mice in which all cells and their descendants that have ever expressed F4/80 express the TdTomato lineage tag, and co-stained the lungs for F4/80 expression to assess their current state of F4/80 expression. We found that at birth, the vast majority of F4/80-lineage^{+} interstitial macrophages expressed F4/80 antigen, as expected, but by the end of the third postnatal week few F4/80-lineage^{+} interstitial macrophages expressed F4/80 (Fig. 5F). This suggests that F4/80 expression turns off in interstitial macrophages postnatally, whereas expression of the permanent F4/80 lineage tag is retained. However, at P4 we noted a transient 'spike' in an interesting interstitial macrophage subpopulation that expressed F4/80 but not the F4/80 lineage tag; this was rapidly followed by a transient increase and subsequent decrease in interstitial macrophages expressing both F4/80 and the F4/80 lineage tag (Fig. 5F).

Taken together, these data suggest two important postnatal transitions in F4/80 interstitial lung macrophages. First, resident interstitial macrophages that derive from embryonic F4/80 macrophages, which expressed F4/80 earlier in postnatal life, turn off F4/80 expression during the first 3 weeks of postnatal life but retain expression of the permanent F4/80 lineage tag. Second, in

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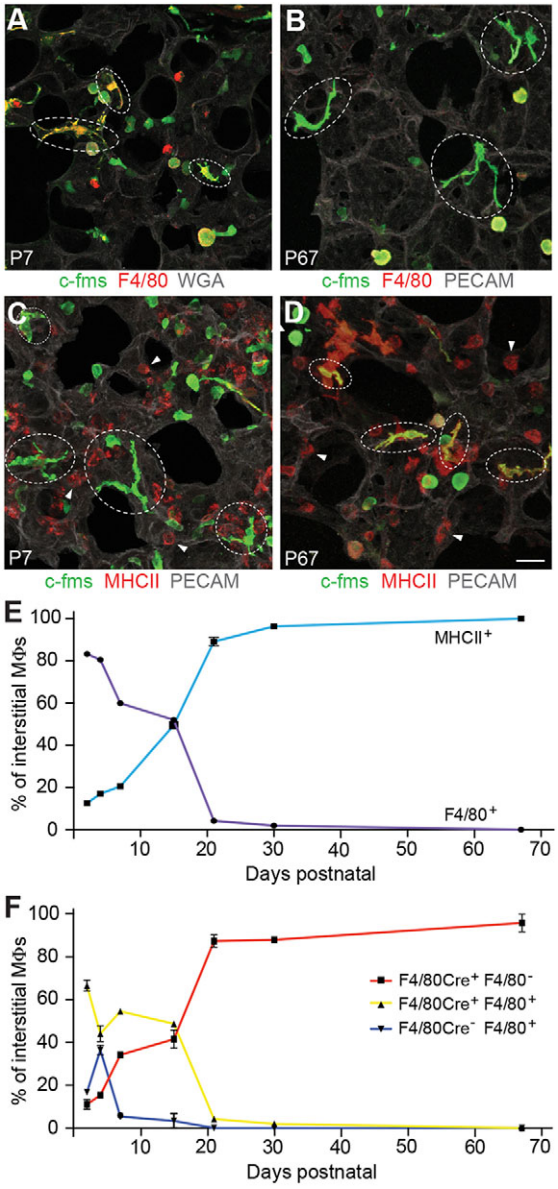

Fig. 5. Gene expression changes in interstitial macrophages after birth. (A-D) Sections of c-fms-EGFP mouse lungs at the indicated postnatal ages immunostained for either F4/80 (A,B) or MHCII (C,D) and wheat germ agglutinin (WGA; A) to show epithelium or PECAM (B-D) to show blood vessels. Because c-fms is a robust and specific macrophage marker, whereas F4/80 is not always macrophage restricted, only c-fms-EGFP^{+} cells were considered macrophages and the identity of the F4/80^{+} c-fms^{-} cells was not determined. Interstitial macrophages (encircled) were distinguished from alveolar macrophages by their location within the walls separating alveolar lumens, and their distinctive dendritic-like morphology. Arrowheads indicate examples of type II alveolar epithelial cells, which express MHCII (Debbabi, 2005) but not c-fms (C,D). Scale bar: 20 µm. (E) Quantification of postnatal changes in abundance of F4/80^{+} and MHCII^{+} interstitial macrophages. Values shown are mean±s.e.m. of *n*=100-210 interstitial macrophages scored in two or three lungs at each time point. Note diminution in F4/80^{+} population and concomitant increase in MHCII^{+} population in the first 3 weeks of life. A small transient postnatal population of interstitial macrophages that are F4/80^{-} and MHCII^{-} is not depicted in the graph. (F) Quantification of postnatal changes in abundance of F4/80^{+} and F4/80Cre-lineage-positive interstitial macrophages. Note the spike at P4 in F4/80^{+}, F4/80Cre-lineage-negative interstitial macrophages (blue line). Values shown are mean±s.e.m. of *n*=90-170 interstitial macrophages scored in two or three lungs at each time point; many of the error bars are small and obscured by the data point symbols. Mϕs, macrophages.

early postnatal life there is a transient influx of a new population of macrophages – a population that only recently turned on F4/80 (F4/80^{+}, F4/80 lineage^{-}) – that soon after express the F4/80 lineage tag (F4/80^{+}, F4/80 lineage^{+}), before downregulating F4/80 expression (F4/80^{-}, F4/80 lineage^{+}) later in postnatal life like the resident (embryonic-derived) F4/80 macrophages.

Parabiosis shows some replacement of interstitial macrophages from the circulation

To determine whether interstitial macrophages can be replenished from circulating cells in the adult, we performed parabiosis experiments in which the circulatory systems of wild-type and transgenic ubiquitous EGFP mice were surgically joined. After 1 or 4 months, we examined the lungs of the wild-type 'recipient' mouse for labeled cells from the EGFP^{+} 'donor'. We found EGFP^{+} donor cells throughout the recipient lung (Fig. 6A), comprising ∼13% of parenchymal interstitial lung macrophages at 1 month and ∼17% at 4 months (Fig. 6B). Thus, circulating progenitors contribute to the interstitial macrophage population during adult life. By contrast, almost no labeled donor cells (<1%) were found within the alveolar compartment (Fig. 6B) after 1 or 4 months of parabiosis. This suggests that alveolar macrophages are not maintained by circulating precursors, consistent with previous reports (Hashimoto et al., 2013; Shibata et al., 2001; Guilliams et al., 2013).

Mac2 macrophages become alveolar macrophages that are maintained independently of the circulation

To determine the postnatal fate of embryonic Mac2 macrophages, we first examined the distribution and localization of Mac2 macrophages in sections of postnatal lungs during the first week of life. At birth, almost all Mac2 macrophages were interstitial (Fig. 7A), like F4/80 macrophages. However, in contrast to F4/80 macrophages, which remain interstitial through adulthood, Mac2 macrophages began to populate the alveoli in the first week of postnatal life. As they did, the interstitial Mac2 macrophage population declined, and by the end of the first postnatal week, almost all Mac2 macrophages were in alveoli (Fig. 7B, 'lumenal'). This implies that there is a regulated entry of Mac2 macrophages from the interstitium into the alveolar space during the first week of postnatal life. In the adult, all alveolar macrophages express Mac2, and Mac2 is expressed almost exclusively on alveolar macrophages (Fig. S2; Table S1).

To determine whether Mac2 macrophages share an origin with the yolk sac-derived interstitial lung macrophage lineage, we examined lungs of the *Runx1*$^{Cre/wt}$: *Rosa26*^{TdTomato} lineage trace mice described above, which effectively labels this interstitial macrophage lineage. We found that Runx1 lineage tracing did not result in substantial labeling of either embryonic (Fig. 3) or adult Mac2 alveolar macrophages (Fig. 4). Similarly, in the parabiosis experiments described above, which gave 17% labeled bone marrow-derived interstitial macrophages in the recipient lung, there were few (∼0.5%) labeled alveolar macrophages in the recipient (Fig. 6). Thus, the Mac2 alveolar macrophage population represents a separate lineage from both yolk sac- and bone marrow-derived interstitial macrophages.

DISCUSSION

Our results show that macrophages of different origins colonize the lung in three successive waves during development, each population eventually localizing to a distinct microanatomical niche in the adult (Fig. 8). The first wave (embryonic F4/80 lineage macrophages) arises from yolk sac precursors and begins at E10.5 with the cells

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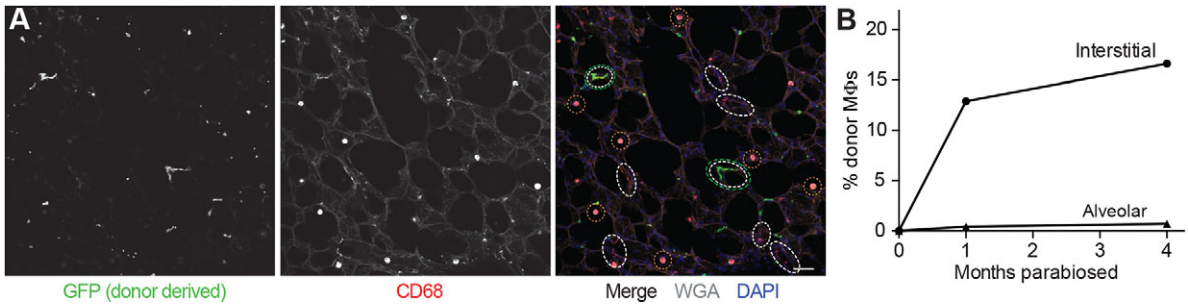

Fig. 6. Replenishment of interstitial but not alveolar macrophages during parabiosis. (A) Lung of a host (unlabeled) C57BL/6J P98 adult mouse after parabiosis for 1 month with a litter mate donor C57BL/6 CAG-EGFP mouse that expresses EGFP (green) ubiquitously. Lung was immunostained for CD68^{+} (all macrophages) and WGA (epithelium) and counterstained with DAPI. Note that some interstitial CD68^{+} macrophages are GFP labeled and hence donor derived (encircled green and white in the merge image), whereas the rest of the interstitial macrophages (encircled white in the merge image) and all alveolar macrophages (encircled red in the merge image) are host derived. Scale bar: 40 µm. (B) Quantification of host interstitial and alveolar macrophages that are donor derived (GFP^{+}) following parabiosis. Note replenishment of interstitial but not alveolar macrophages. Values shown are from *n*=130-160 donor macrophages scored in one animal per time point. Mϕs, macrophages.

spreading uniformly throughout the lung interstitium during embryonic development. The second wave (embryonic Mac2 macrophages) begins 2 days later and arises from a separate source, probably the fetal liver as suggested by adoptive transfer of fetal liver monocytes (Guilliams et al., 2013) and their appearance in the lung shortly after hematopoiesis initiates there (Morrison et al., 1995). Like F4/80 macrophages, they distribute throughout the lung interstitium during embryonic development, intermingled with F4/80 macrophages. However, during the first week of postnatal life they enter the alveoli, where they remain and sustain themselves as alveolar macrophages. The distribution of the embryonic F4/80 lineage macrophages also changes dramatically during the first week of life, restricting from diffuse interstitial localization to selective localization in submesothelial and perivascular zones; we call these 'primitive' interstitial lung macrophages because of their origin early in development (primitive hematopoiesis) before initiation of definitive hematopoiesis, and in parallel with yolk sac-derived microglia (Ginhoux et al., 2010). During this same period, a third wave of macrophages that we call 'definitive' interstitial macrophages arrives and diffusely populates the lung interstitium, replacing the parenchymal embryonic F4/80 lineage population. The definitive interstitial macrophages are maintained by circulating progenitors.

The results show that the distinct populations of alveolar and interstitial macrophages are established very early in lung development and maintained throughout life. In contrast to the classical model of interstitial macrophages as precursors to alveolar macrophages, our data imply that alveolar and interstitial macrophages are separate lineages and cell types with distinct origins and kinetics, which are localized and maintained in separate microanatomical compartments in the lung, presumably by signals specific to each lineage. Although previous studies did not find yolk sac-derived macrophages in the adult lung (Guilliams et al., 2013; Epelman et al., 2014), our data identify a small but distinct population of interstitial lung macrophages ('primitive') that derive from the yolk sac and localize preferentially to perivascular and peripheral regions rather than being intermixed with the bone marrow-derived 'definitive' interstitial macrophages.

We presume that these lung macrophage lineages have different functions. The differences between alveolar and interstitial macrophages have long been recognized, with alveolar macrophages involved in the phagocytosis of foreign particles and surfactant catabolism within alveoli, and interstitial macrophages involved in tissue remodeling and maintenance, antigen presentation and dendritic cell functions to prevent airway allergy. But the functional distinctions between the two interstitial macrophage populations are less clear. The two interstitial populations appear similar morphologically and share similar molecular phenotypes, based on the small number of markers examined so far. Nevertheless, the specific spatial localization of the

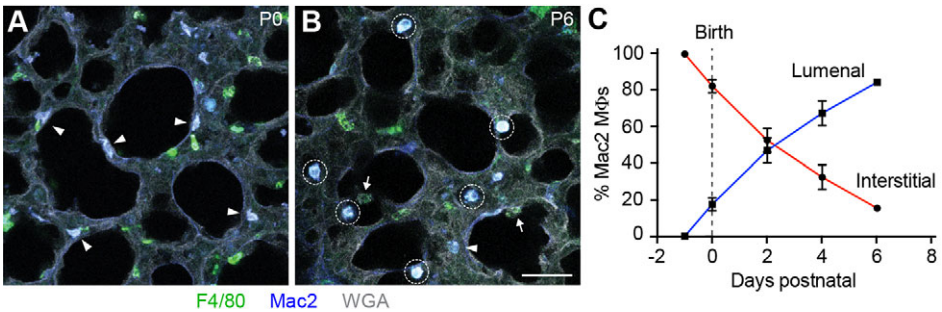

Fig. 7. Mac2 macrophages populate the alveoli in the first week after birth. (A,B) Sections of lungs from wild-type mice at the indicated postnatal ages that were immunostained for macrophage markers F4/80 and Mac2, and WGA (epithelium). At birth (A), nearly all Mac2 macrophages are interstitial (arrowheads) but by P6 (B) nearly all Mac2 macrophages are within alveoli (circles), with only a few still interstitial (arrowhead). F4/80 macrophages (green) remain interstitial: inspection of serial optical sections confirmed that the occasional F4/80 macrophage that in a single optical section can appear lumenal (arrows) are actually interstitial. At the stages shown, Mac2 macrophages express low, variable levels of F4/80 as well as WGA. Scale bar: 40 µm. (C) Quantification of Mac2 macrophage localization one day before birth (E18.5) and during first week after birth. Values shown are mean±s.e.m. for *n*=140-450 macrophages scored in three sections of each lung at each time point; some error bars are very small and obscured by the data point symbols.

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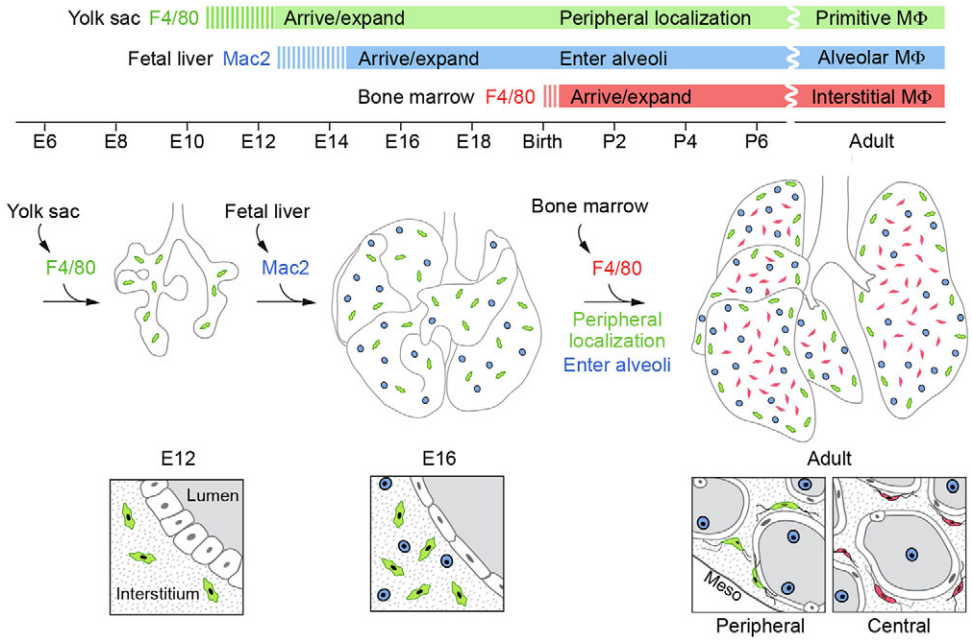

Fig. 8. Model of developmental origin of lung macrophage diversity. Timeline (top) and schematics (middle) of lungs at E12, E16, and in the adult illustrating the major developmental events and global localization of the three lung macrophage lineages shown in green, blue and red. Schematics at the bottom are close-ups showing interstitial and lumenal localization of the macrophage lineages, with airway epithelial cells indicated in white, the airway lumen in gray and the interstitium stippled. F4/80 embryonic macrophages (green) arrive from yolk sac in the earliest wave beginning at E10.5. Two days later, Mac2 embryonic macrophages (blue) begin arriving, most likely from the fetal liver (Guilliams et al., 2013). During the first week of postnatal life, Mac2 macrophages enter the lumen to become alveolar macrophages, which self-renew. F4/80 embryonic macrophages persist at specific locations (peripheral and perivascular) to become the 'primitive' interstitial macrophages, whereas those in the parenchyma are replaced by a new population of 'definitive' interstitial macrophages (red) from the circulation, presumably of bone marrow origin. Both F4/80 lineages turn off F4/80 and turn on MHCII during the first 3 weeks of postnatal life (not shown). Meso, mesothelium; MΦ, macrophage.

primitive (yolk sac-derived F4/80) interstitial macrophages to peripheral and perivascular regions of the lung demonstrates that they retain some biological distinction from the broadly distributed definitive interstitial macrophages. Interestingly, perivascular and peripheral regions have recently been shown to be relative 'hot spots' of alveolar renewal and adenocarcinoma tumor foci (Desai et al., 2014), suggesting that the primitive interstitial macrophages might promote or be attracted to stem cell activity, perhaps by growth factors that the cells in the region secrete. Each population might also differentially influence regional tissue homeostasis, immunology and pathology.

Dramatic changes occur during the first week of postnatal life in all three lung macrophage populations. The embryonic F4/80 and Mac2 macrophage populations are intermingled during embryonic development but remain distinct, then both populations undergo changes that, although different, occur simultaneously. The Mac2 embryonic macrophages, which are nearly all interstitial at the end of embryonic development, are nearly all lumenal by the end of the first postnatal week, suggesting regulated entry into the developing alveoli. During this same period, the F4/80 embryonic macrophages remain interstitial but preferentially localize to the peripheral and perivascular regions, whereas the definitive interstitial macrophages enter the lung and localize diffusely throughout the interstitium. The remarkable synchrony with which these three transitions occur predicts tight temporal and spatial regulation of the specific signals controlling localization of the three lung macrophage lineages. Tight spatial control of macrophage localization continues in the adult with circulating precursors replenishing the pool of interstitial macrophages without displacing the peripheral F4/80 (primitive) macrophage lineage or entering the alveolus to replenish alveolar macrophages. However, upon depletion of alveolar macrophages by irradiation or toxin (Blussé van Oud Alblas et al., 1981; Godleski and Brain, 1972; Landsman et al., 2007), or following lung transplantation (Thomas et al., 1976), alveolar macrophages can be replenished from the circulation, perhaps through reactivation of the fetal liver lineage or conversion of one of the other lineage progenitors. A top priority now is to identify the specific signals that control localization and maintenance of each macrophage lineage, and to understand how these signals change during development and are altered following injury or disease, and if this leads to reactivation or interconversion of macrophage progenitor pools.

A mosaic of macrophages of three different origins populates the lung. Our results show that these are not simply a heterogeneous mix of interconvertible macrophages of different origins. Rather, each macrophage lineage arrives in the lung at a different time, destined to become a specific type of macrophage with a unique microanatomical niche and renewal mechanism. Although the lung with its complex microarchitecture, physiology and primary exposure to environmental pathogens could be unique with its spatially and functionally distinct macrophage lineages, we expect that functionally distinct lineages will also be found in other organs. There is already a suggestion of such diversity among macrophages populating the different layers of the spleen, including red pulp, marginal zone, marginal metallophilic and white pulp macrophages (den Haan and Kraal, 2012). Although genetic lineage tracing has not yet been done, each subpopulation appears to replenish with different kinetics following depletion (van Rooijen et al., 1989), and self-renewal appears to be limited to white pulp and metallophillic macrophage populations (Wijffels et al., 1994). For other tissues, most recent studies have focused on a single macrophage

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population, without describing or distinguishing between populations in close proximity but different microanatomical domains. In the skin, the focus has been on epidermal Langerhans cells, which were found to arise from the fetal liver with a minority from the yolk sac but replaceable by bone marrow-derived cells. Although the developmental origin of macrophages may not be crucial in the skin (Hoeffel et al., 2012), it remains possible that important distinctions exist in the localization and function of Langerhans cells of different origin. Similarly, for heart macrophages, a number of phenotypically distinct subpopulations have been identified, each apparently composed of a mix of macrophages of different origins, including yolk sac and fetal liver (Epelman et al., 2014), but apparently replaceable during inflammation by bone marrow-derived cells. However, the lineage relations between the different subpopulations are unclear, as are their specific spatial localizations, so here too close inspection could reveal macrophage subpopulations of distinct lineages that do not interconvert.

For each organ, it will be important to determine the number of macrophage lineages that are present, to map their locations down to the microanatomical niche, and to ascertain their distinct functions, mechanisms of maintenance and any conditions that induce lineage interconversion. This would also provide insight into the roles of each lineage in specific diseases and how they might be controlled or replenished.

MATERIALS AND METHODS

Mice

Wild type was C57BL/6J. The c-fms-EGFP (Rae et al., 2007), *Runx1-MER-Cre-MER* (Samokhvalov et al., 2007; Ginhoux et al., 2010), C57BL/6-Tg(CAG-EGFP)131Osb (abbreviated CAG-EGFP) (Okabe et al., 1997), F4/80 Cre (Schaller et al., 2002) and TdTomato Cre reporter (Madisen et al., 2010) mice were described previously.

Inducible lineage labeling

In the *Runx1-MER-Cre-MER* experiments, pregnant females received an intraperitoneal injection of 2 mg tamoxifen (Sigma-Aldrich, T5648) dissolved in corn oil (Sigma-Aldrich, C8267). Injections were performed at E6.75. Embryos were harvested at the stages indicated. For long-term lineage labeling, litters were born and animals euthanized at the ages indicated. The labeling of yolk sac progenitors by tamoxifen injection at E6.75 was confirmed by control experiments that showed numerous macrophages carrying the Runx1 lineage label in adult brain and heart, tissues previously demonstrated to contain yolk sac-derived macrophages (Epelman et al., 2014; Ginhoux et al., 2010). Tamoxifen injection 0.5 or 1 day later also labeled yolk sac progenitors, but these later injections were not used here because they resulted in substantially increased labeling of Mac2 macrophages. Early tamoxifen injection commonly results in spontaneous abortion and maternal hemorrhage, so biological replicates were limited (n=2) for these experiments.

Lung collection, fixation and processing

Embryos were staged by vaginal plugging of the mother, with noon on the day of appearance of the plug taken as E0.5. Individual embryos were also staged by fetal crown-rump length at time of euthanasia. Lungs were removed *en bloc* and fixed for 30 min to 1 h in 4% paraformaldehyde (PFA) in PBS at 4°C. Postnatal mice were euthanized by carbon dioxide inhalation, the abdominal aorta was severed, and sternotomy was performed. PBS (Ca^{2+}- and Mg^{2+}-free, pH 7.4) was gently perfused into the right ventricle by manual pressure using a syringe with a 21-gauge needle to clear the pulmonary vasculature. For mice up to 7 days old, lungs were removed *en bloc* and fixed in 4% PFA for 1-2 h at 4°C. For mice 8 days or older, lungs were collected and processed as above except that following clearance of the pulmonary vasculature the ventral trachea was incised and cannulated with a blunt needle that was secured in place by tying a suture around the trachea. Lungs were then gently inflated to full capacity with molten low melting point agarose (Sigma-Aldrich, 2% in PBS) after which the cannula was withdrawn and the suture tightened to prevent leakage. Ice-cold PBS was dripped into the thorax to solidify the agarose, then the inflated lungs were removed *en bloc* and processed as above. They were then fixed for 2-7 h at 4°C. All lungs were cryoprotected in 30% sucrose overnight at 4°C then submerged in OCT (Tissue Tek) in an embedding mold, frozen on dry ice, and stored at −80°C. Sections of 30 and 50 µm thickness obtained using a cryostat (Leica CM3050S) were collected on glass slides and stored at −80°C after curing at room temperature overnight.

Parabiosis

For parabiosis experiments, female CAG-EGFP mice expressing EGFP ubiquitously under a chicken β-actin/rabbit β-globin hybrid promoter were surgically conjoined to female wild-type littermates to generate a common anastomosed circulatory system. Parabiosis was performed as previously described (Kamran et al., 2013) and in accordance with the guidelines established by Stanford University for the humane treatment of animals. Matching skin incisions were made from the olecranon to the knee joint of each mouse, and the subcutaneous fascia was bluntly dissected to create about 0.5 cm of free skin. The olecranon and knee joints were attached by a single 2-0 silk suture and tie, and the dorsal and ventral skins were approximated by staples or continuous suture. Because the procedure causes substantial morbidity and mortality, we investigated only two different periods of parabiosis (1 and 4 months; Fig. 6), and because these gave consistent results we did not perform biological replicates.

Lung fluorescence immunostaining and microscopy

Lungs were sectioned at 30-50 µm using a Leica CM3050S cryostat. Tissue sections were immunostained using a the following primary antisera and dilutions: chicken anti-GFP (Abcam, ab13970; 1:1000), rat anti-F4/80 (clone CI:A3-1; Serotec; 1:500, purified, MCA497G; 1:300, FITC-conjugated MCA497FA), rat anti-Mac2 (clone M3/38; Cedarlane; 1:500, purified, CL8942AP; 1:200, biotinylated, CL8942B), rat anti-CD68 (clone FA-11; Serotec, MCA1957; 1:500), rat anti-MHCII (clone M5/114; Millipore, MABF33; 1:500), rabbit anti-Lyve1 (Abcam, ab14917; 1:500; polyclonal), rat anti-MARCO (clone ED31; Serotec, MCA1849; 1:500), rat anti- rat anti-MMR (clone MR5D3; Serotec, MCA2235GA; 1:500), hamster anti-CD11c (clone N418; R&D Systems, MAB69501; 1:250), Armenian hamster anti-PECAM (clone 2H8; Serotec, MCA1370Z; 1:250), rat anti-E-cadherin (clone ECCD-2; Life Technologies 13-1900; 1:500), rabbit anti-E-cadherin (clone 24E10; Cell Signaling, 3195S; 1:250), rabbit anti-dsRed (for tdTomato; Clontech, 632496; 1:500; polyclonal). Biotinylated primary antibodies were detected by tyramide signal amplification (TSA, Plus Fluorescein System; PerkinElmer, NEL741001KT). Secondary antibodies were all used at 1:500.

Confocal images were acquired using either a Leica Sp7 or a Zeiss LSM 780 microscope. Images were processed using LCS and LAS AF software (Leica), Zen Microscope and Imaging Software (Zeiss) and ImageJ. For high-resolution images of a large field, stitching of tiled scans with 15% overlap was performed using the Zen software at the time of image acquisition. Adobe Photoshop was used for overlays and image processing.

Quantification of embryonic lung macrophages

To determine the number of Mac2 and F4/80 macrophages per lung (Fig. 2E), embryonic lungs were isolated, fixed, and serially sectioned as above, and the cross-sectional area of each section through the entire lung was measured using ImageJ software, and multiplied by section thickness to obtain section volumes, which were summed to obtain total lung volume. Representative cross-sections (n=3 or 4) were then stained for Mac2 and F4/80, macrophages were scored, and the total number in the lung calculated by dividing by the volume ratio of the scored sections to the entire lung. This morphometric approach was not possible for postnatal lungs, because inflation of postnatal lungs is variable, giving non-uniform lung density.

Quantification of abundance and distribution of Runx1 lineage macrophages

Sections of lungs of *Runx1-MER-Cre-MER* ($Runx1^{Cre/wt}$); $Rosa26^{TdTomato/TdTomato}$ mice of various ages, induced with tamoxifen at

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E6.75, were imaged as described above. Because macrophage density appears uniform throughout the interstitium, we estimated the percentage of interstitial macrophages that derived from the Runx1 lineage from the ratio of the area populated by Runx1 lineage-labeled cells over the entire interstitial area in a cross-section of a lung lobe, using ImageJ to trace and calculate the respective areas. From each mouse analyzed in this way (n=2), three cross-sections of a single lobe were analyzed. To estimate the peripheral bias in the distribution of Runx1 lineage-labeled macrophages, the proportion of the peripheral area (defined as fewer than five alveolar layers away from the mesothelial lining) that was occupied by Runx1-lineage macrophages divided by the proportion of the parenchymal area (all other parenchymal regions, excluding areas within large airways and vessels) occupied by Runx1-lineage macrophages was calculated, analyzed in the sections as described above.

Acknowledgements

The authors thank Drs David Hume, Igor Samokhvalov and Tushar Desai for mouse strains; members of the Krasnow laboratory for valuable discussions and comments on the manuscript; and Maria Petersen for help preparing figures and the manuscript.

Competing interests

The authors declare no competing or financial interests.

Author contributions

S.Y.S.T. and M.A.K. designed the experiments, performed data analysis and wrote the manuscript. S.Y.S.T. performed the experiments.

Funding

This work was supported by a fellowship award from the American College of Surgeons Foundation (to S.Y.S.T.); a Gallo Fellowship from the Children's Health Research Institute at Stanford University (to S.Y.S.T.); a grant from the National Institutes of Health [U01-HL099995 to M.A.K.]; and an Investigator Award from the Howard Hughes Medical Institute (to M.A.K.). Deposited in PMC for release after 6 months.

Supplementary information

Supplementary information available online at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.129122/-/DC1

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Supplementary Figures

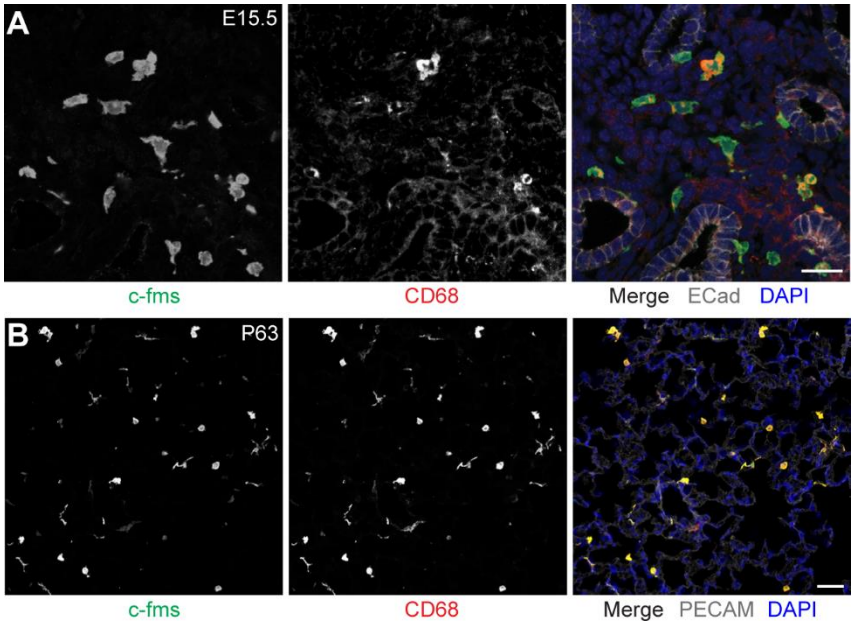

Figure S1. Expression of CD68 in mid-embryonic and adult lung macrophages. **A,B,** Sections of embryonic (E15.5, A) and adult (P63, B) c-fms-EGFP mouse lungs immunostained for GFP (green) to show cytoplasm of c-fms-expressing macrophages, and for macrophage marker microsialin CD68 (red), epithelial marker E-cadherin (grey, **A**) or endothelial marker PECAM (grey, **B**), and counterstained with DAPI (blue, nuclei). CD68 is expressed in all c-fms-EGFP positive macrophages. CD68 expression at E15.5 is speckled and dim (**A**), whereas in the adult it is more prominent (**B**). Bar, 20 um (A), 40 um (B)

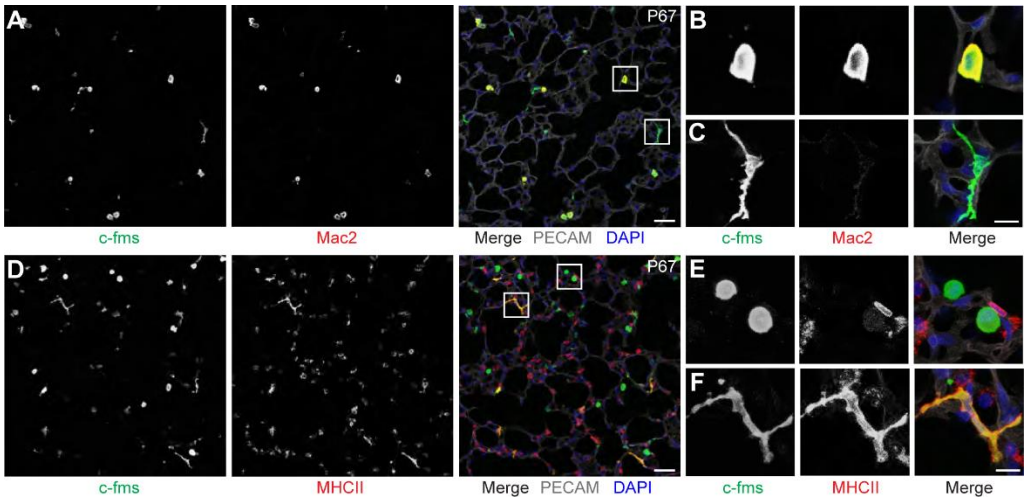

Figure S2. Mac2 and MHCII expression in complementary populations of adult lung macrophages. Sections of adult (P67) c-fms-EGFP mouse lungs immunostained for EGFP (c-fms, green) to show cytoplasm of c-fms-expressing macrophages, and for macrophage markers Mac2 (red, **A-C**) or MHCII (red, **D-F**), endothelial marker PECAM (grey), and counterstained with DAPI (blue). **B** and **C** are close-ups of boxed regions in **A**, and **E** and **F** are close ups of boxed regions in **D**. Note Mac2 is expressed by all of the alveolar macrophages (**A, B**) but none of the interstitial macrophages (**A, C**), and conversely, MHCII is expressed by all of the interstitial macrophages (**D, F**) but none of the alveolar macrophages (**E, F**). Bar, 40 um (A), 10 um (C), 40 um (D), 10 um (F).

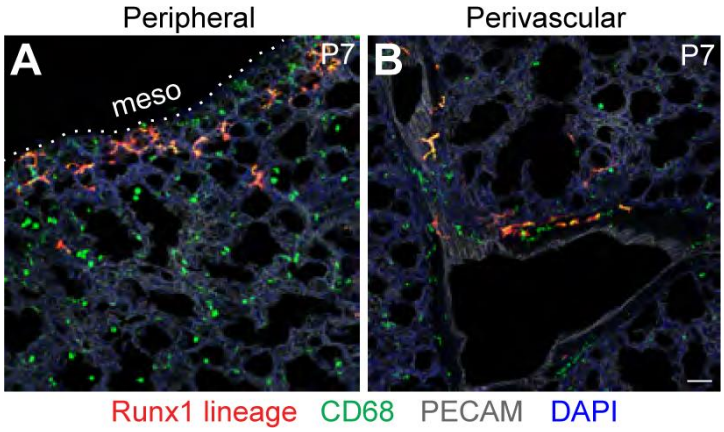

Figure S3. Lineage trace of yolk sac precursors in the juvenile lung. Lineage trace of yolk sac precursors as in Figure 4 using Runx1-CreER>TdTomato (yolk sac) lineage trace (Runx1 lineage, red) induced at E6.75, but lung harvested at juvenile stage (postnatal day P7) and then immunostained for CD68 (green) to visualize all macrophages, for PECAM (blood vessels, grey), and counterstained with DAPI (blue). Peripheral (**A**) and more internal (**B**) lung sections are shown. Note that as in the adult lung (Figure 4), yolk sac derived (Runx1 lineage-labeled) macrophages (yellow) localize to the interstitium peripherally near the mesothelium (meso, **A**) and more internally surrounding a large blood vessel (**B**), indicating that the change from broad interstitial to restricted distribution of yolk sac derived macrophages occurs during the first week of postnatal life. Bar, 40 um

Table S1. Summary of marker expression in lung macrophages

MΦ marker (synonyms)	Protein/function	Marker expression level[a, b]					
		F4/80 MΦ		Int MΦ	Mac2 MΦ		Alv MΦ
		E13.5	E16.5	Adult	E13.5	E16.5	Adult
csf1r-EGFP (c-fms, MacGreen)	Macrophage colony stimulating factor 1 receptor; tyrosine kinase	+++	+++	+++	+++	+++	+++
Cd68	Microsialin; endosomal/lysosomal membrane protein	+ [c]	++ [c]	+++ [c]	+ [c]	++ [c]	+++ [c]
Lyz2 [d] (Lyz)	Lysozyme C type M; secreted bacteriolytic protein	+	+/++	+/++	+++	+++	+++
Emr1 (Adre1, F4/80)	EGF-like, mucin-like hormone receptor-like 1; cell surface glycoprotein	**+++** [c]	++ [c]	- [c]	(few) + [e]	+/++ [e]	+/++ [e]
H2 [d] (MHCII)	H-2 class II histocompatibility agent	-	-	+++ [c]	-	-	-
Lyve1	Lymphatic vessel endothelial hyaluronic acid; membrane protein	(many) +++ [c,f]	(few) +++ [c,f]	-	-	-	-
Lgals3 (Mac2)	Galactose-specific lectin that binds IgE; acute inflammatory responses	- [c]	- [c]	- [c]	+++ [c]	+++ [c]	+++ [c]
Itgax (CD11c)	Integrin alpha-X, receptor for fibrinogen; cell interactions in inflammatory responses	+	+	+	++	+++	+++
Marco	Macrophage receptor; pattern recognition, bacterial-binding	-	-	-	-	(some) ++	(some) +++
Mrc1 (MMR)	Macrophage mannose receptor; endocytosis of glycoproteins	-	-	-	-	+++	+++

[a] +++, high macrophage (MΦ) marker expression; ++, moderate; +, low; -, none

[b] Fraction of macrophages expressing marker: all, no parenthetical note; (many), >60%; (some), 30-60%; (few), <30%

[c] Based on quantitation of >200 *csf1r-EGFP*+ macrophages

[d] Also expressed in alveolar type II cells

[e] Expression on Mac2+ MΦs variable but increases over time

[f] Transient expression in F4/80 macrophages (peaks at E14.5 and largely absent by E16.5)

Abbreviations: MΦ, macrophage; Int, interstitial; Alv, alveolar