

Organ-Specific Fate, Recruitment, and Refilling Dynamics of Tissue-Resident Macrophages during Blood-Stage Malaria

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SUMMARY

Inflammation-induced disappearance of tissue-resident macrophages represents a key pathogen defense mechanism. Using a model of systemic blood-stage malaria, we studied the dynamics of tissue-resident macrophages in multiple organs to determine how they are depleted and refilled during the course of disease. We show that *Plasmodium* infection results in a transient loss of embryonically established resident macrophages prior to the parasitemia peak. Fate-mapping analysis reveals that inflammatory monocytes contribute to the repopulation of the emptied niches of splenic red pulp macrophages and hepatic Kupffer cells, while lung alveolar macrophages refill their niche predominantly through self-renewal. Interestingly, the local microenvironment of the spleen and liver can “imprint” the molecular characteristics of fetal-derived macrophages on newly differentiated bone marrow-derived immigrants with remarkably similar gene expression profiles and turnover kinetics. Thus, the mononuclear phagocytic system has developed distinct but effective tissue-specific strategies to replenish emptied niches to guarantee the functional integrity of the system.

INTRODUCTION

Macrophages are resident in virtually all tissues of the body, forming a network of specialized cells, including alveolar macrophages (lung), red pulp macrophages (spleen), Kupffer cells (liver), microglia (brain), Langerhans cells (epidermis), osteoclasts (bone), and histiocytes (connective tissue). As well as their role in tissue development and homeostasis, macrophages form the first line of defense against pathogens alongside other cells of the innate immune system (Davies et al., 2013; Gupta et al., 2016; Varol et al., 2015). Although macrophages are crucially

involved in mediating protection to malarial parasites mechanistically through phagocytosis, clearance of infected erythrocytes, antibody-dependent cell inhibition, and cytokine production, they can also contribute to severe immunopathology, such as cerebral malaria and placental malaria (Chua et al., 2013).

The majority of tissue-resident macrophages are established prenatally, maintaining their numbers *in situ* by self-renewal without any major hematopoietic input (Ginhoux and Guillems, 2016; Perdiguero and Geissmann, 2016; Sheng et al., 2015). However, under certain circumstances, such as low-grade inflammation (Bain et al., 2014), mechanical stress (Lavine et al., 2014), genotoxic injury (Beattie et al., 2016), and infection (Blériot et al., 2015; Robinson et al., 2012), the resident macrophage population can be refilled by infiltrating monocytes from the circulation. These infiltrating cells are able to differentiate into tissue-resident macrophages closely resembling their fetal counterparts at both a phenotypic and transcriptional level (Epelman et al., 2014; Scott et al., 2016).

In particular, depletion of resident macrophages driven by inflammation, a phenomenon commonly known as the macrophage disappearance reaction (Barth et al., 1995), renders the niche available and accessible to infiltrating monocytes, which rapidly refill the emptied compartment (Guillems and Scott, 2017). Macrophage disappearance mediated by inflammatory cell death, also called necroptosis, has been characterized in enteroinvasive bacterial infections, such as *Salmonella enterica*, *Listeria monocytogenes*, and *Shigella flexneri* (Blériot et al., 2015; Robinson et al., 2012; Zychlinsky et al., 1992). Necroptosis of tissue-resident macrophages can occur as an endpoint of the host innate immune response, involving pathogen uptake, release of inflammatory mediators, and subsequent recruitment of circulating monocytes and neutrophils. Alternatively, as was recently suggested by Ginhoux et al. (2017), macrophage necrosis itself can be an active feature of the innate antibacterial immune response, preceding and initiating the recruitment of bactericidal cells, and can therefore be viewed as altruistic macrophage “suicide.”

Infection-mediated necroptosis caused by non-bacterial microorganisms, such as viruses, parasites, or fungi, has not yet been systematically studied. Using a model of acute blood-stage malaria, we therefore sought to determine the impact of



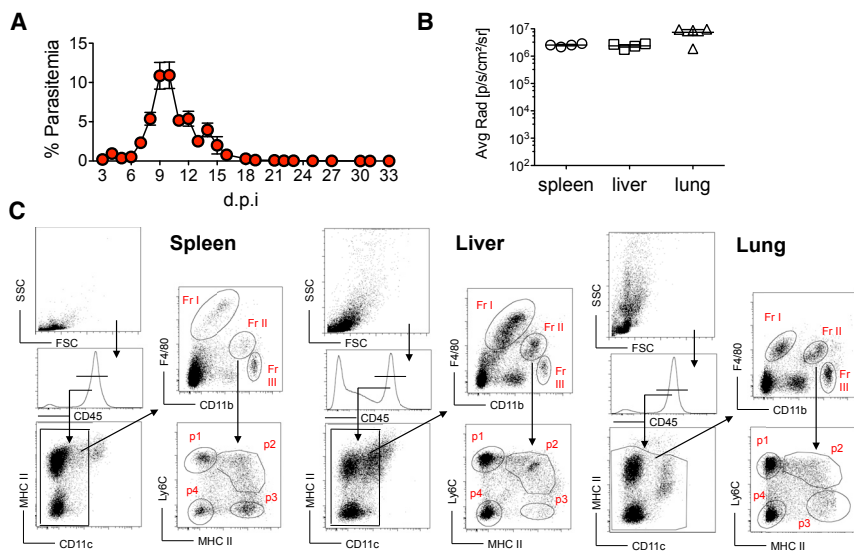


Figure 1. *P. yoelii* Blood-Stage Infection in *Kit^{MerCreMer/R26}* Mice and Myeloid Cell Populations in the Spleen, Liver, and Lungs

(A) Course of parasitemia during 17XNL *P. yoelii* infection in *Kit^{MerCreMer/R26}* mice. Chart shows percentage of parasite-infected red blood cells (iRBCs) over time. Data shown are representative of five independent experiments with $n = 4-6$ mice/group, 6–10 weeks old. Error bars represent SEM. (B) Bioluminescence imaging of lungs, liver, and spleen at day 9 of *P. yoelii* infection. Average radiance (p/sec/cm²/sr) values (log) of four mice are shown. (C) Representative flow cytometry dot plots of myeloid cell subpopulations in the spleen, liver, and lungs in uninfected animals. Gating strategy omitted the initial FSC and SSC gate for an unbiased analysis of all myeloid cell populations. Three different myeloid fractions (Fr I–III) are defined by the differential expression of F4/80 and CD11b. Fr I represents F4/80^{hi} tissue-resident macrophages. Fr II cells expressing F4/80^{int} contain monocytes (p1) and two monocyte-derived macrophage subpopulations (p2 and p3) based on differential expression of MHCII and Ly6C. Fraction III cells, which lack F4/80 expression, represent the neutrophils.

non-bacterial infection on tissue-resident macrophage disappearance and refilling. Blood-stage malaria represents a useful model because parasitic infection occurs systemically and affects multiple organs, including the spleen, liver, and lungs. These organs are known to be seeded under steady-state conditions by embryonic-derived self-renewing tissue-resident macrophages with minimal contribution from adult circulating monocytes (Guilliams and Scott, 2017; Perdiguero and Geissmann, 2016; Sawai et al., 2016; Sheng et al., 2015).

We show that prior to the peak of *Plasmodium* infection, there is a rapid disappearance of resident macrophages. Although newly recruited inflammatory monocytes could partially replenish the red pulp macrophages and Kupffer cell compartment (open niche), they were unable to refill the alveolar macrophage compartment, which remained inaccessible to monocytes during infection (closed niche). Furthermore, following parasite clearance, cells in the “refilled” niches, regardless of their origin, closely emulated the characteristics of the original cell population.

RESULTS

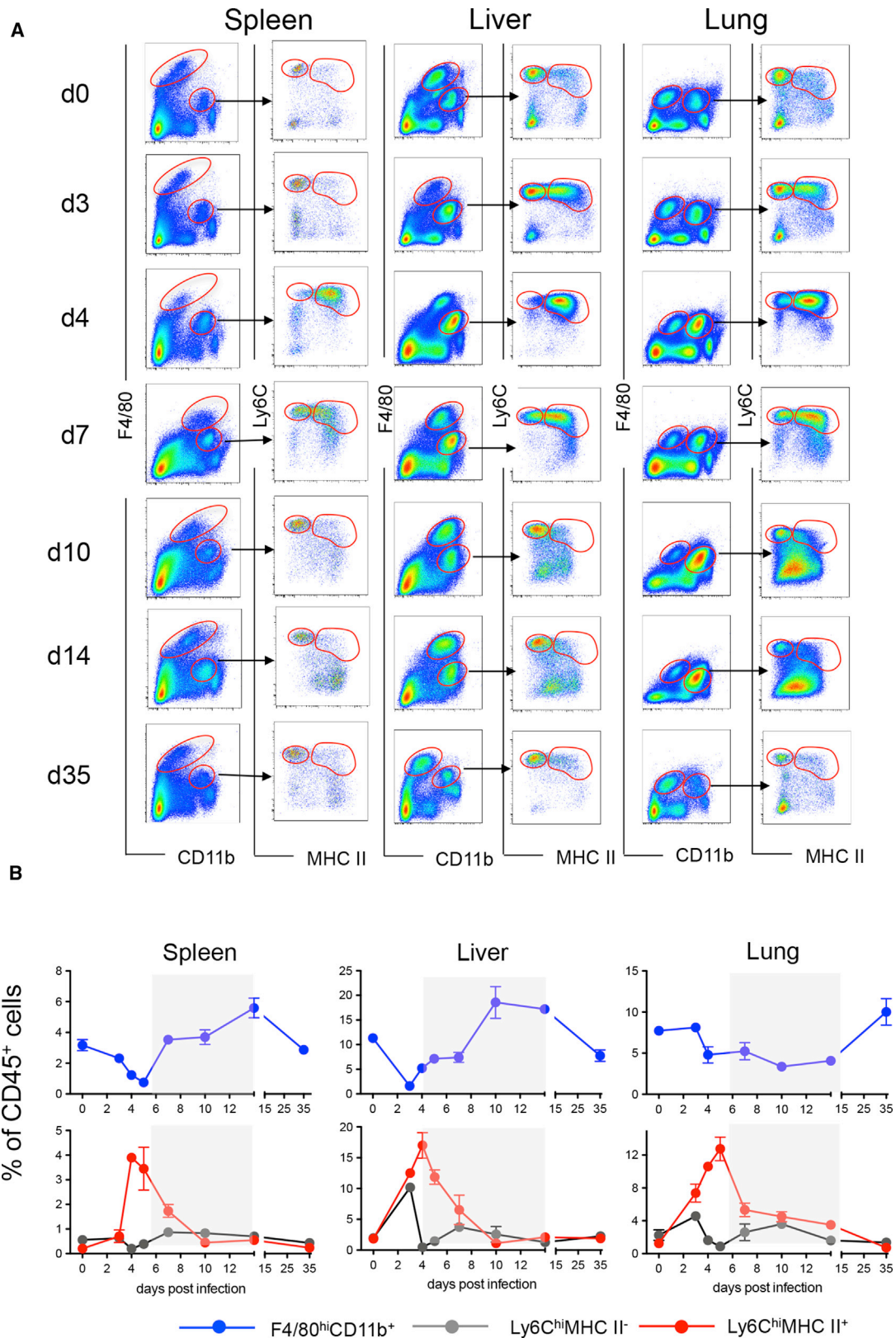
Following *P. yoelii* Infection, Tissue-Resident Macrophages Decline Concomitant with Recruitment of Inflammatory Monocytes

Blood-stage non-lethal 17XNL *P. yoelii* infection causes self-resolving acute malaria in mice. A progressive increase of parasite-infected red blood cells (iRBCs) is observed in the blood from 4 days post-infection (d.p.i.), reaching its highest levels between 9 and 10 d.p.i., before the induction of parasite-specific T cell and antibody responses, which rapidly control and ultimately clear the infection (Freeman and Parish, 1981; Jayawardena et al., 1975). The parasite is not limited to the blood circulation and can be sequestered into the capillaries and venules of different organs (Kaul et al., 1998; Schofield and

Grau, 2005). At 9 d.p.i., parasite can be detected in perfused spleen, liver, and lungs as shown by quantitative bioluminescent analysis using a *P. yoelii* parasite (*Py17XNL-Luc*) expressing the reporter protein luciferase (Figure 1B).

The involvement of multiple organs during *P. yoelii* infection allowed us to investigate the consequences of parasite sequestration on tissue-specific myeloid cell populations during the different stages of infection until final clearance. Malaria causes acute transient tissue inflammation and local production of hemozoin, which is rapidly engulfed by monocytes and macrophages, increasing their total cell volume greatly (Olivier et al., 2014). We therefore performed an unbiased flow cytometry analysis, gating only on CD45⁺ total leukocytes and omitting the usual forward scatter (FSC) and side scatter (SSC) pre-gating. This strategy therefore included analysis of all cell sizes, including FSC/SSC^{high} cells that may be otherwise be discounted (Figure 1C). In the absence of infection, F4/80^{hi}CD11b^{lo} tissue-resident macrophages (Fr I), as well as the classical F4/80^{int}CD11b^{hi} monocytes and monocyte-derived macrophages (Ly6C^{hi}MHCII⁻ [p1], Ly6C^{hi/int}MHCII⁺ [p2], and Ly6C⁻MHCII⁺ [p3]), are found in the spleen, liver, and lungs (Figures 1C and 2A, top). F4/80⁻CD11b^{hi} neutrophils (Fr III) and F4/80^{int}CD11b^{hi} Ly6C⁻MHCII⁻ eosinophils are also present.

Upon *P. yoelii* infection, the myeloid landscape undergoes dramatic fluctuations. At the very early stages of infection (3–4 d.p.i.), when the parasite is almost undetectable in the blood by flow cytometry, we detected a massive infiltration of monocyte-derived cells, which co-express Ly6C and MHC II (p2), in all three organs (Figures 2A, 2B, and S1). After the peak of infiltration (6–10 d.p.i.), inflammatory Ly6C^{hi}MHCII^{hi} monocytes then declined to basal levels, although more slowly in the lungs (Figures 2A, 2B, and S1). In contrast, at 3–4 d.p.i., the numbers of F4/80^{hi}CD11b^{lo} (Fr I) tissue-resident macrophages rapidly dropped (Figures 2A, 2B, and S1). This reduction was most apparent in hepatic F4/80^{hi}CD11b^{lo} Kupffer cells at 3 d.p.i. but



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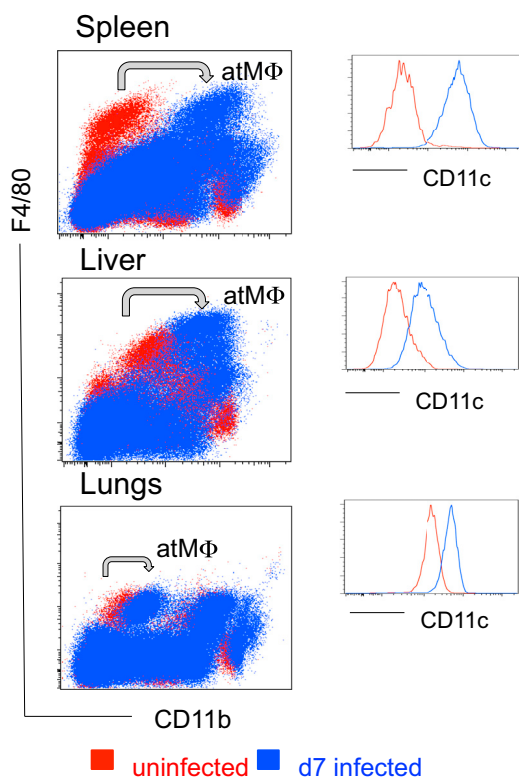


Figure 3. Appearance of Transient-Activated F4/80^{hi} Resident Macrophages during *P. yoelii* Infection

Flow cytometry dot plots (CD11b versus F4/80) show an overlay of spleen, liver, and lung cells obtained at day 0 (red) and day 7 post-infection (blue) (left). On the right, histograms show an overlay of CD11c-labeled spleen, liver, and lung cells obtained at day 0 (red) and day 7 post-infection (blue). Overlay graphs shown are representative of the experimental data from Figure 2 with 3 or 4 mice/group.

was also observed in spleen red pulp macrophages and lung alveolar macrophages (at 4 d.p.i.). F4/80^{hi}CD11b^{lo} cells disappeared completely when conventional FSC and SSC gating was used. However, when SSC^{high} cells were included, the remaining Fr I cells transited in the F4/80^{hi} SSC^{high} macrophage population in all three tissues tested. These cells expressed high levels of CD11b and CD11c (Figure 3) and were hence called activated transient macrophages. This transient population persisted for several days (Figure 2B, gray shaded area) but disappeared upon resolution of the infection (Figure 2B).

Tissue-resident macrophages regained their original frequency, although with distinct organ-dependent kinetics. In the

liver, numbers of Kupffer cells were quickly restored from day 4 onward, whereas the spleen red pulp macrophage population was restored 1 week after infection (Figure 2B, left and middle). Despite the massive infiltration of inflammatory monocytes into the lungs, the reduction of alveolar macrophages was not as extensive as observed in the liver and spleen. However, restoration was slow, and cells did not return to their original numbers until 14 d.p.i. (Figure 2B, right). Once the infection was cleared, tissue-resident macrophages (spleen F4/80^{hi} red pulp macrophages, hepatic Kupffer cells, and lung alveolar macrophages) regained their original frequency and phenotype.

High-Dimensional Analysis Visualizes Myeloid Cell Dynamics during Blood-Stage Malaria

To allow high-resolution and unbiased data-driven dissection of the dynamics of the myeloid populations, we performed PhenoGraph cell clustering (Levine et al., 2015) and uniform manifold approximation and projection (UMAP) analysis, a fast and reliable non-linear dimensionality reduction technique (McInnes and Healy, 2018; Becht et al., 2018). We analyzed a dataset of 81 samples enriched for CD11b⁺F4/80⁺ myeloid cells obtained from uninfected and *P. yoelii*-infected spleen, liver, and lungs at different time points throughout infection. Ten parameters, including light scatter, were incorporated to quantify and visualize the diversity and evolution of the tissue-resident myeloid cells. Spleen and liver samples were analyzed together as they exhibited similar phenotype; lung samples were analyzed separately (Figures 4 and S2). In the case of the spleen and liver, UMAP analysis revealed 32 distinct PhenoGraph cell clusters representing non-activated and activated stages of myeloid cell subpopulations, including resident F4/80^{hi} macrophages (distributed over clusters 1, 2, 8, 21, and 31), Ly6C⁺MHCII⁻ monocytes (clusters 12 and 25), inflammatory Ly6C⁺MHCII⁺ (clusters 9 and 10), CD11b⁺F4/80⁻ neutrophils (cluster 32), and eosinophils (cluster 20), during the different stages of malarial infection (Figure 4, left). In the lungs, PhenoGraph analysis and UMAP visualization segregated 30 clusters with a distinct alveolar macrophage cluster (clusters 15 and 25), as well as Ly6C⁺MHCII⁻ monocytes (clusters 6), inflammatory Ly6C⁺MHCII⁺ (clusters 8 and 18), CD11b⁺F4/80⁻ neutrophils (cluster 2), and eosinophils (cluster 19) (Figure 4, right).

Color-mapped expression plots of F4/80, Ly6C (Figures 4B and 4C; blue to red, low to high), and CD11c (Figures S2A and S2B) over the time course of infection further confirmed the cell dynamics observed by flow cytometry. A massive influx of inflammatory Ly6C⁺MHCII⁺ monocytes was apparent at the early stages of infection in all three organs (Figures 4B and 4C, Ly6C), returning to basal levels after parasite clearance. Concurrently,

Figure 2. Transient Ablation of Tissue-Resident Macrophages and Concomitant Inflammatory Monocyte Infiltration during Non-lethal *P. yoelii* Blood-Stage Infection

(A) Representative flow cytometry dot plots of F4/80^{hi} macrophages (Fr I), Ly6C⁺MHCII⁻ and Ly6C⁺MHCII⁺ monocyte Fr II subpopulations in the spleen, liver, and lungs in uninfected and *P. yoelii*-infected (day 0 to day 35) Kit^{MerCreMer/R26} mice.

(B) Graphical representation of the percentage of F4/80^{hi} macrophages (top, blue curve), Ly6C⁺MHCII⁻ (bottom, gray curve), and Ly6C⁺MHCII⁺ monocytes (bottom, red curve) during the time course of *P. yoelii* blood-stage infection. Each value represents the percentage of the correspondent cell population ± SEM (3 or 4 mice/group per time point). The same results were obtained in BALB/c WT mice. Gray shaded area indicates the presence of transient-activated macrophages (atMΦ).

See also Figure S1.

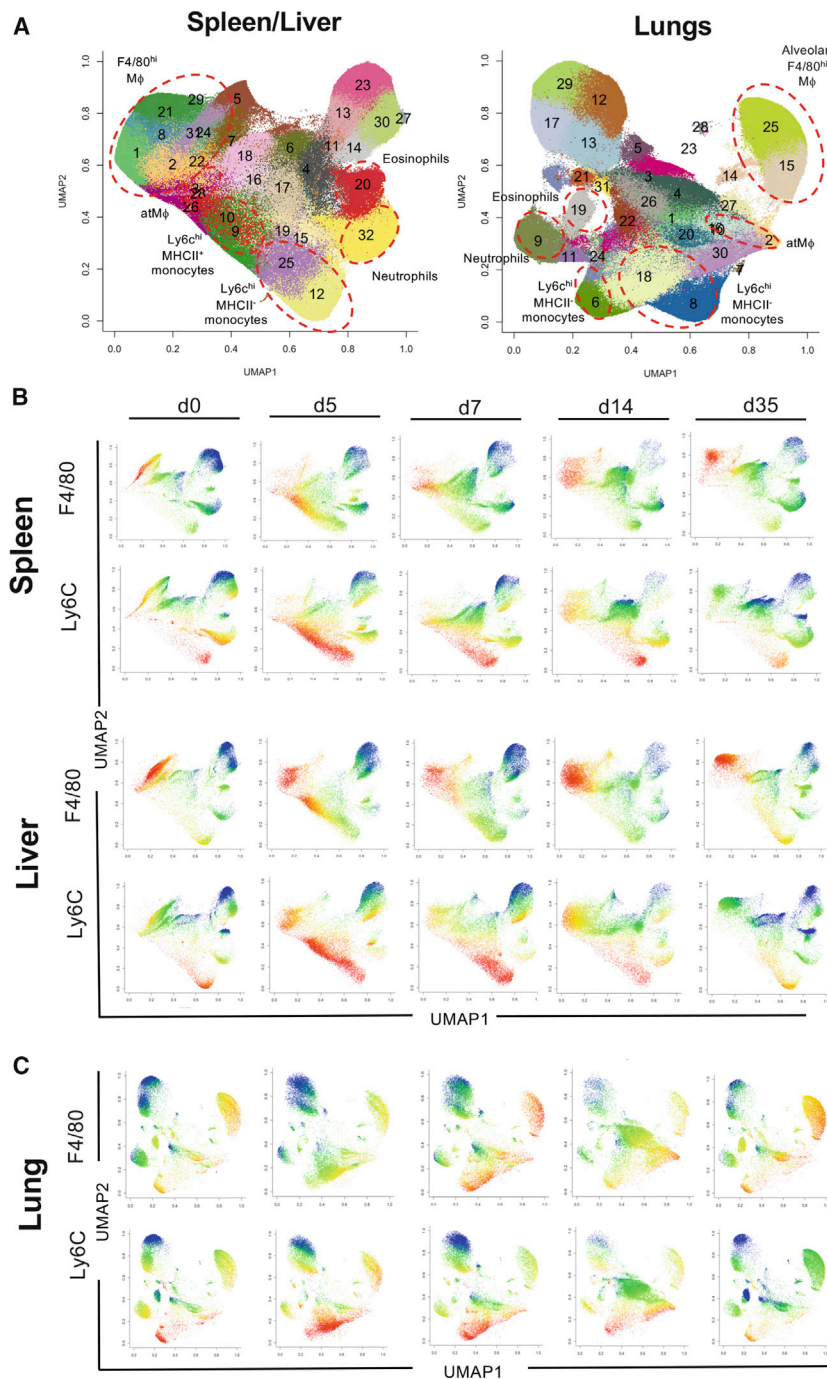


Figure 4. High-Dimensional Analysis of Myeloid Cell Populations during *P. yoelii* Infection

(A) Two different UMAP projections of F4/80⁺CD11b⁺ myeloid cells from *P. yoelii*-infected and uninfected mouse spleens and livers (left) and lung cells (right) with annotated cell clusters based on the expression of eight different surface markers. (B and C) Expression plots of F4/80 and Ly6C color-mapped from blue (low expression) to red (high expression) in different organs. (B) spleen (top) and liver (bottom) and (C) lungs. Each time point shown is representative of 3 mice/group. See also Figures S2 and S3.

The Majority of Fetal-Derived F4/80^{hi} Kupffer and Red Pulp Macrophages, but Not Alveolar Macrophages, Are Replaced by Bone Marrow-Derived Progenitors during *P. yoelii* Blood-Stage Infection

Most tissue-resident macrophages are derived from fetal progenitors where the numbers are maintained through self-renewal. However, when the niche becomes available, for example, through diphtheria toxin (DT)-mediated depletion, bone marrow (BM)-derived monocytes have been shown to give rise to self-renewing and fully differentiated Kupffer cells in the liver (Scott et al., 2016) and dermal macrophages in the skin (Baranska et al., 2018).

To understand how local macrophage populations are refilled following systemic, blood-stage *P. yoelii* infection and to monitor the infiltration of BM-derived cells, we exploited a Kit^{MerCreMer/R26} fate-mapping mouse whereby YFP labeling could be induced in early hematopoietic progenitors in adult mice via tamoxifen treatment. Tamoxifen-treated Kit^{MerCreMer/R26} mice were infected with blood-stage *P. yoelii*, and YFP labeling was monitored by flow cytometry over the course of the infection until 35 d.p.i. in the liver, spleen, and lungs (Figure 5A). The YFP labeling of BM-derived monocytes (p1), inflammatory monocytes (p2), and neutrophils (used as a 100% reference because of their short population half-time and constant replacement by BM progenitors) into the

resident F4/80^{hi} macrophage numbers decreased over the first days followed by a transient increase in CD11c⁺ macrophage numbers (clusters 3 and 28 in spleen and liver and clusters 2 and 10 in lungs) (Figures S2A and S2B). Interestingly, at day 35 post-infection, spleen and hepatic F4/80^{hi} macrophages (clusters 1, 8, and 21) do not overlap with their uninfected counterparts (cluster 31), as a result of their higher SSC profiles (Figure S2C), most likely caused by the accumulation of malarial hemozoin (Figure S3).

spleen, liver, and lungs was analyzed in control uninfected and *P. yoelii*-infected Kit^{MerCreMer/R26} mice and compared with YFP labeling of tissue-resident F4/80^{hi} macrophages.

Ly6C^{hi}MHCII⁻ monocytes (p1) and Ly6C^{hi}MHCII^{hi} inflammatory monocytes (p2) remained fully labeled (relative to neutrophil numbers) during the course of infection in all three organs, reflecting their rapid turnover. In the spleen and liver, YFP labeling in the tissue-resident F4/80^{hi} macrophage population could be detected only from 5 d.p.i., gradually increasing to 70%–80%

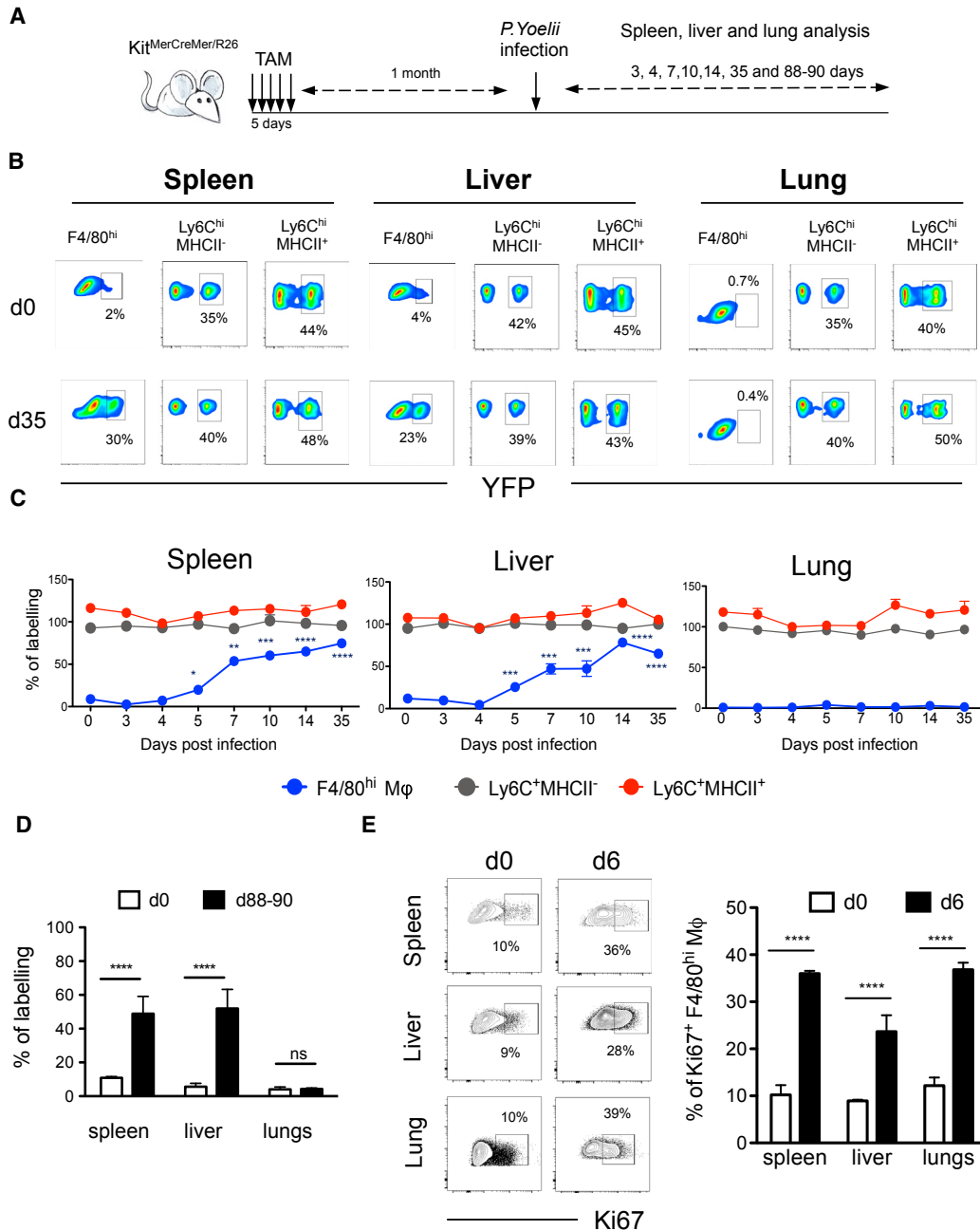


Figure 5. Fetal-Derived F4/80^{hi} Liver and Spleen Macrophages, but Not Alveolar Macrophages, Are Replaced by BM Progenitors during *P. yoelii* Blood-Stage Infection

(A) Schematic representation of the adult fate-mapping protocol using Kit^{MerCreMer/R26} mice. Mice (8–10 weeks old) were injected with tamoxifen five times, and groups of four to eight animals were infected with *P. yoelii*. At distinct time points after infection, mice were sacrificed and analyzed for the presence of YFP labeling.

(B) Representative flow cytometry analysis indicating the labeling efficiency of distinct myeloid cell populations in the spleen, liver, and lungs: Fr I tissue-resident F4/80^{hi} macrophages, Fr II p1 monocytes, and Fr II p2 monocyte-derived cells. The labeling of neutrophils (not shown) acted as internal controls for labeling efficiency and the tracings are from the same mouse. Representative dot plots (uninfected [day 0] and 35 days post-infection) showing YFP labeling percentage. (C) Line charts representing the mean percentage of YFP⁺ cells after normalization to neutrophils over different time points of *P. yoelii* infection in the spleen, liver, and lungs. Blue line, F4/80^{hi} macrophages; gray line, Ly6C⁺MHC II⁻ monocytes; red line, Ly6C⁺MHC II⁺ monocytes. Error bars represent the SEM of three or four individual mice from two independent experiments.

(D) Long-term persistence of BM-replenished macrophages in spleen and liver detected at days 88–90 post-infection: YFP labeling percentage of steady state (white bars) and days 88–90 post-infection macrophages (black bars). Error bars represent the SEM of four individual mice.

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relative to neutrophil YFP labeling by 35 d.p.i., suggesting that the majority of F4/80^{hi} tissue-resident macrophages following parasite clearance are newly derived from the BM (Figures 5B and 5C, left and middle, and Figure S4). Even at later time points, around day 90 post-infection, both spleen and liver F4/80^{hi} macrophages were still YFP labeled suggesting long-term persistence of BM-replenished macrophages in this acute malaria infection model (Figure 5D). Although dramatically reduced in numbers during the initial phases of infection, the remaining macrophages showed increased Ki67 staining. They therefore also contributed in refill of spleen and liver macrophage compartments through self-renewal (YFP^{neg} fraction) (Figure 5E).

In contrast, almost no YFP⁺ cells were detectable in the fraction of F4/80^{hi}CD11b^{lo} macrophages in the lung throughout the course of *P. yoelii* infection, indicating that BM-derived monocytes do not contribute to niche refilling, whereas infiltrating monocytes and inflammatory monocytes showed strong YFP labeling throughout (Figures 5B and 5C, right, and Figure S4).

As shown in Figure 2, the disappearance of F4/80^{hi} macrophages was not as drastic in the lungs compared with the spleen and liver, despite comparable levels of parasite infection (Figure 1B), with approximately 50% of alveolar macrophages remaining at their lowest point (Figure 2). These residual cells were therefore able to gradually refill the compartment by self-renewal (Figures 2 and S6). In fact, alveolar macrophages, isolated at 6 d.p.i., showed an increased expression of the cellular proliferation marker Ki67 in comparison to the steady-state counterparts (Figure 5E). This suggested enhanced self-renewal in order to refill the emptied macrophage compartment.

Post-entry Monocytes Gain the Characteristics of Fetal-Derived Macrophages

To investigate the molecular relationship between the original fetal-derived macrophage population and the immigrant macrophage population following *P. yoelii* infection, we performed RNA sequencing (RNA-seq) on tissue-resident macrophages from the spleen, liver, and lungs of uninfected and recovered *P. yoelii*-infected BALB/c mice at 35 d.p.i. Hierarchical clustering and principal-component analysis (PCA) revealed that resident F4/80^{hi} macrophages isolated from uninfected and infected tissues clustered closely together (Figures 6A and 6B), indicating a high similarity between the pre- and post-infection macrophage populations. Alveolar macrophages, which were replaced predominantly by self-renewal, displayed the highest similarity (~1.7% differentially expressed genes [DEGs]) between the pre- and post-infection groups. But even in the spleen and liver, in which we detected large numbers of infiltrating monocytes into the F4/80^{hi} macrophage niche, we observed a low degree of DEGs pre- and post-infection (3.7% and 2.9%, respectively). Thus, newly immigrating cells were able to adopt the gene expression patterns of the original fetal-derived population within the F4/80^{hi} macrophage niche. These slight changes of

surface phenotypes between steady-state and post-infection macrophages did not reflect major differences in ontogeny but instead in their cellular activation. In fact, DEGs related to expression of MHC class II and invariant chain as well as an inflammation-dependent scavenger receptor, CD163L1 (Moeller et al., 2012), were always higher in post-infection macrophages in all tested organs (Figures 6C and S5). However, as sorted spleen and liver macrophages obtained at day 35 post-infection included a fraction of monocyte-derived macrophages together with embryonically seeded macrophages, the imprint of true “embryonic” DEGs could thereby be weakened.

Because of the high similarity between pre- and post-infection resident macrophages (Figure 6), we tested whether the turnover kinetics of the monocyte-refilled liver and spleen macrophages differed from the “original” self-renewing, fetal-derived counterparts in uninfected organs. Again, exploiting our Kit^{MerCreMer/R26} fate-mapping mouse, tamoxifen was administered 2 months after *P. yoelii* infection (Figure 7A). The levels of YFP labeling of Kupffer cells and splenic red pulp macrophages in control uninfected and infected recovered mice was almost identical (Figure 7B), indicating that upon resolution of malarial infection, the replenished compartments of both liver Kupffer cells and splenic red pulp macrophages regained similar turnover kinetics as their self-maintaining fetal counterparts.

DISCUSSION

As a first line of defense, tissue-resident macrophages are anatomically well positioned across a wide range of lymphoid and non-lymphoid organs (Mowat et al., 2017) to capture potential invading pathogens. Therefore, maintenance of the numbers, locations, and functionality of these cells must be tightly regulated to enable an optimal early immune response. Using blood-stage malaria as an experimental model, we performed detailed studies of the tissue-specific fate, recruitment, and refilling dynamics of tissue-resident macrophages during the course of infection in distinct organ niches. We recently highlighted the crucial role of CD169⁺ tissue-resident macrophages in containing the parasite burden, thereby limiting infection-induced inflammation (Gupta et al., 2016). Here, we report that tissue-resident macrophages respond to systemic parasite infection in an organ-specific manner with characteristic death, refilling, and turnover rates (Figure S6).

In the liver, resident Kupffer cells, positioned on the sinusoidal endothelial cells, form the first line of hepatic immune cells, sensing and orchestrating responses against blood-borne toxins, bacteria, and parasites (Krenkel and Tacke, 2017). The strategic location of Kupffer cells therefore enables them to sense malaria iRBCs adhering to the hepatic vascular endothelium from the very early stages of infection. Already at 3 d.p.i., in the presence of subpatent parasitemia, we observed disappearance of Kupffer cells concomitant with a massive

(E) F4/80^{hi} Macrophages increase their self-renewing activity during *P. yoelii* infection. Spleen, liver, and lung macrophages collected at days 0 and 6 post-infection were intracellularly stained with anti-Ki67. Representative dot plots (left) and bar charts (right) representing the percentage of Ki67⁺ macrophages. Error bars represent the SEM of three individual mice.

Statistical significance was determined by nonparametric two-tailed Mann-Whitney U test for two groups: *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001. See also Figure S4.

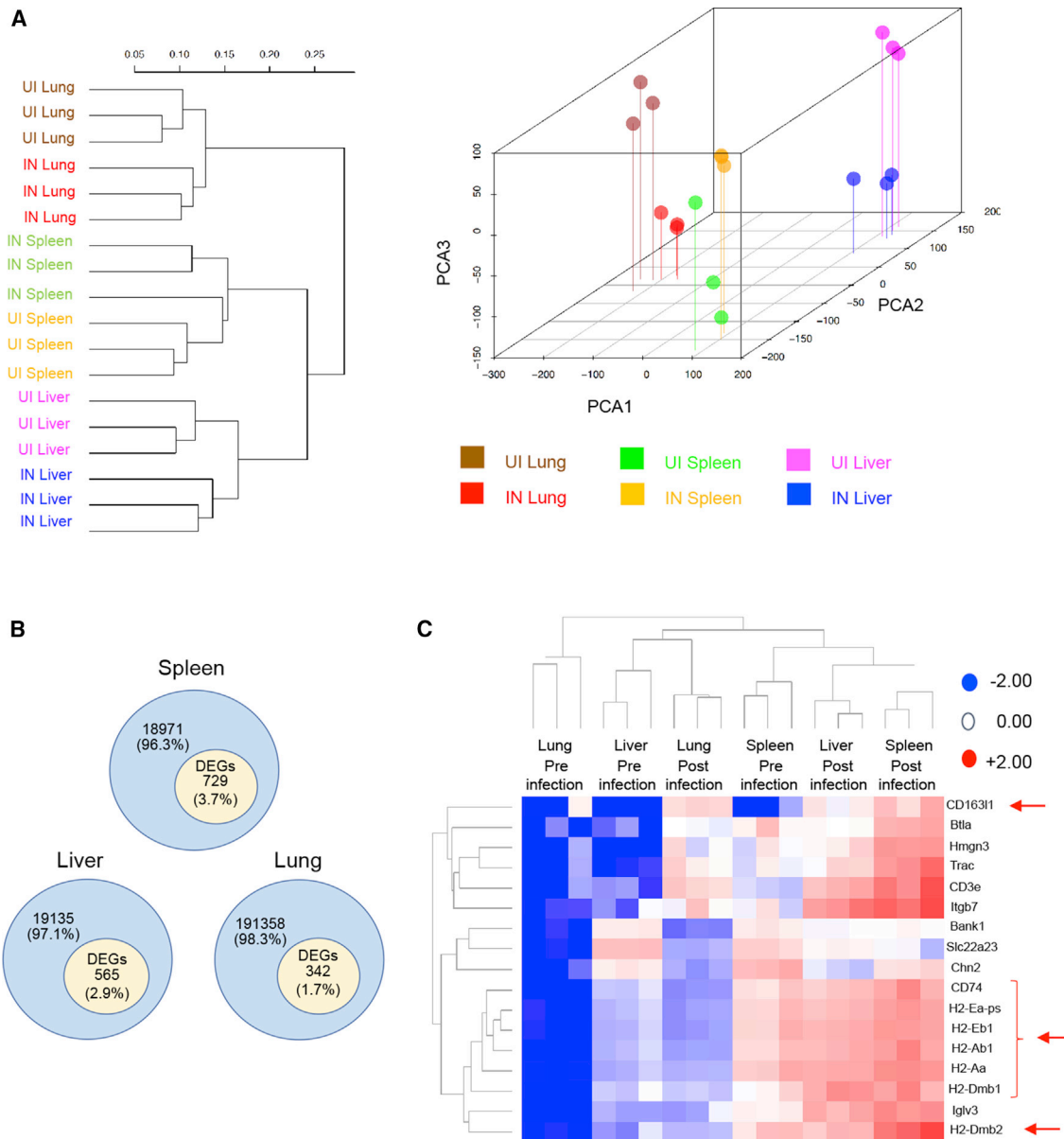


Figure 6. Transcriptomic Analysis Reveals High Similarity between Pre- and Post-infection Tissue-Resident macrophages.

Transcriptome analysis of spleen, liver, and lung tissue-resident macrophages isolated and purified from uninfected and *P. yoelii*-infected animals (n = 8–10, 35 days post-infection [d.p.i.]).

(A) Hierarchical clustering and three-dimensional principal-component analysis (PCA) of spleen, liver, and lung F4/80^{hi} tissue-resident macrophages before and after *P. yoelii* infection. Hierarchical clustering and PCA were performed with log₂-transformed FPKM (fragments per kilobase of transcript per million mapped reads) values.

(B) Number of genes tested and percentage of DEGs between pre- and post-infection spleen, liver, and lung tissue-resident macrophages. Comparisons of pre- and post-infection macrophages for each of the tissues were performed using limma. DEGs were selected using Benjamini-Hochberg adjusted p values of <0.05.

(C) Top 50 shared DEGs between spleen, liver, and lung (uninfected day 0 versus post-infection day 35). Red arrows indicate DEG hits of interest. Heatmaps were generated with row/gene normalized Z scores and log₂ fold expression change represented from –2 (blue) to +2 (red). See also Figure S5.

recruitment of inflammatory monocytes. These BM-derived inflammatory cells were able to refill the emptied niche gradually from day 4 onward with an activated transient macrophage subpopulation. The activated transient macrophage population we observed in the liver during malarial infection closely resembles

recent reports of transient hepatic macrophage populations observed in a number of pathophysiological conditions causing red blood cell damage, such as hemolytic anemia and sickle-cell disease, where they are thought to be responsible for rapid erythrocyte removal and iron recycling (Theurl et al., 2016).

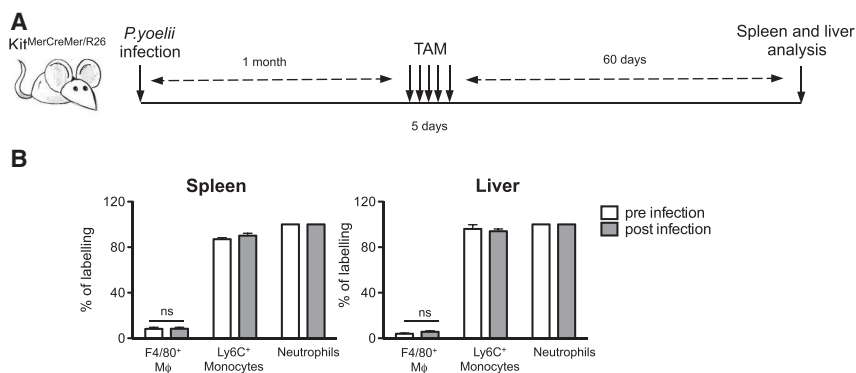


Figure 7. Turnover Kinetics of the Monocyte-Refilled Liver and Spleen Macrophages Do Not Differ from the “Original” Embryonic-Derived Counterparts

(A) Schematic representation of the adult fate-mapping protocol using pre-infected Kit^{MerCreMer/R26} mice. Mice (8–10 weeks old) were infected with *P. yoelii*. Tamoxifen was injected intraperitoneally (i.p.) on 5 consecutive days from 30 d.p.i., and mice were sacrificed 60 days later and analyzed for the presence of YFP-positive cells.

(B) Following a similar gating strategy shown in Figure 2, YFP labeling was analyzed in spleen and liver tissue-resident macrophages, monocytes, and neutrophils. Bar charts represent the mean percentage of YFP⁺ cells after normalization to

neutrophils. White bars, uninfected; gray bars, 95 d.p.i. Error bars represent the SEM of six individual mice from two independent experiments. Statistical significance was determined by nonparametric two-tailed Mann-Whitney U test for two groups. ns, non-significant.

Following parasite clearance, the BM-derived monocytes adopted the characteristics of “original” Kupffer F4/80^{hi}CD11b^{lo} cells with a similar phenotype and turnover rate. Our results are in good agreement with those of Scott et al. (2016), who also showed a small number of DEGs and close PCA clustering of monocyte-derived and steady-state Kupffer cells in a DT depletion model (Scott et al., 2016).

In our malaria model, despite the fast clearance of the parasite, a considerable fraction of BM-derived YFP⁺ F4/80^{hi} cells persisted at 90 d.p.i., unlike in the model of stressed erythrocyte challenge in which the monocyte-derived Kupffer cells showed faster kinetics of replacement (Theurl et al., 2016). This discrepancy could be explained by the prolonged inflammation caused by long-term deposition of hemozoin (Frita et al., 2012; Levesque et al., 1999), an inflammatory, crystallized by-product of hemoglobin catabolism (Olivier et al., 2014), released in the circulation during the *Plasmodium* infection (Boura et al., 2013). The presence of a moderate inflammation in affected organs was confirmed by our RNA-seq analysis. Newly entered macrophages in spleen and liver showed a slightly activated phenotype, as did also the locally self-renewing alveolar macrophages, suggesting that the tissue microenvironment and not their origin imprints the phenotype of the resident macrophages during the post-infection period.

Splenic red pulp macrophages, located between the splenic cords and venous sinuses, where they are readily accessible to blood, are well positioned to clear iRBCs and have been implicated in controlling blood-stage malaria (Borges da Silva et al., 2015). As observed for Kupffer cells, *Plasmodium* infection triggered red pulp macrophages disappearance alongside a massive infiltration of inflammatory monocytes from 4 d.p.i., 1 day later than in liver. An inflammatory splenic monocyte population, similar to what we observe here, was described during *P. chabaudi* infection, where the monocytes acted as an early anti-parasitic mechanism (Sponaas et al., 2009). Here, fate-mapping studies revealed efficient refilling of emptied niches by BM-derived monocytes, which gradually differentiated into F4/80^{hi}CD11b^{lo} cells. The differentiation to a tissue-resident macrophage phenotype occurred in competition with a small proportion of surviving self-renewing resident macrophages.

The alveolar macrophage compartment has previously been reported to remain closed, with minimal contribution of monocytes under steady-state conditions in adult mice (Guilliams et al., 2013). We have extended these observations under inflammatory conditions, demonstrating that the loss of the alveolar macrophage compartment was compensated by self-renewal of surviving cells and not by monocytes, despite the massive parasite burden and marked infiltration of monocytes in the lungs. This is most likely because the alveolar niche is inaccessible to monocytes that cannot penetrate through the alveolar epithelium.

The observed cellular dynamics during *Plasmodium* infection favor the recently proposed “beneficial cell suicide” model whereby homeostatically important cells are replaced by cells better equipped to fight against infection (Ginhoux et al., 2017). Remarkably, the system is very sensitive. Both inflammatory monocytes and resident macrophages are able to sense small numbers of iRBCs, suggesting that the “reaction” is possibly triggered indirectly through inflammatory mediators and not through the physical phagocytosis of iRBCs. Sequestered iRBCs are known to produce malaria-specific bioactive products, such as glycosylphosphatidylinositol (GPI), which act as pathogen-associated patterns (PAMP) to initiate the secretion of inflammatory cytokines and the upregulation of vascular endothelium adhesion molecules (Schofield and Hackett, 1993; Schofield et al., 1996; Tachado et al., 1997). In addition, several parasite inflammatory toxins are released at the time of iRBC rupture, including malaria hemozoin pigment (Boura et al., 2013; Corbett et al., 2015; Olivier et al., 2014), TatD-like DNase (Chang et al., 2016), and a tyrosine-tRNA synthase (Bhatt et al., 2011), which trigger macrophage and monocyte activation, in absence of high parasite loads.

In summary, our data underline the dynamic but tissue-specific nature of monocyte and tissue-resident macrophage networks during *Plasmodium* infection. These coordinated systems provide an effective innate immune response against invading pathogens, while moderating inflammation-induced tissue damage, and ensure that the functional integrity of the tissue-resident macrophage network is maintained following systemic infection.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- **KEY RESOURCES TABLE**
- **CONTACT FOR REAGENT AND RESOURCE SHARING**
- **EXPERIMENTAL MODEL AND SUBJECT DETAILS**
 - Mice and ethics statement
- **METHOD DETAILS**
 - Parasite strain, infection protocol and disease assessment
 - Tamoxifen-inducible fate-mapping mouse model in Kit^{MerCreMer/R26} mice
 - Cell isolation and flow cytometry
 - Transcriptomics analysis by RNA-sequencing and bioinformatics analysis
- **QUANTIFICATION AND STATISTICAL ANALYSIS**
 - Cell subpopulations analysis
 - High-dimensional flow cytometry data analysis
 - RNaseq analysis
- **DATA AND SOFTWARE AVAILABILITY**

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures and can be found with this article online at <https://doi.org/10.1016/j.celrep.2018.11.059>.

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AUTHOR CONTRIBUTIONS

Conceptualization, C.R.; Methodology, S.M.L., J.S., P.G., and F.Z.; Investigation, S.M.L., and J.S.; Formal Analysis, S.M.L. and C.R.; Bioinformatic Analysis, K.D.; PhD Supervision, L.R.; UMAP High-Dimensional Analysis, E.W.N.; Writing – Original Draft, C.R.; Writing – Review & Editing, C.R. and K.K.; Visualization, C.R.; Funding Acquisition, C.R.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-mouse CD11c (clone: N418), PE-Cy7	BioLegend	Cat. # 117318; RRID: AB_493568
Anti-mouse CD11b (clone: M1/70), BUV395	BD Bioscience	Cat. # 563553
Anti-mouse CD45 (clone: 30F11), APCFire	BioLegend	Cat. # 103154; RRID: AB_2572116
Anti-mouse Ly6C (HK1.4), BV605	BioLegend	Cat. # 128036; RRID: AB_2562352
Anti-mouse Ly6G (clone: 1A8), FITC	BioLegend	Cat. # 127606; RRID: AB_1236494
Anti-mouse F4/80 (clone: BM8), PE	BioLegend	Cat. # 123112; RRID: AB_893482
MHCII (clone:M5/114.15.2), BV421	BioLegend	Cat. # 107632; RRID: AB_2650896
Ki67 (clone: SolA15), eFluor660	eBioscience	Cat. # 50-5698; RRID: AB_2574234
Bacterial and Virus Strains		
Parasite: <i>Plasmodium yoelii</i> Py1.1-GFP	Laurent Renia Lab (A*STAR, Singapore)	Tarun et al., 2007
Parasite: <i>Plasmodium yoelii</i> Py17XNL-Luc	Peter Preiser Lab (NTU, Singapore)	Unpublished data
Chemicals, Peptides, and Recombinant Proteins		
Tamoxifen	Sigma-Aldrich	Cat. # T5648
Corn oil	Sigma-Aldrich	Cat. # C8267
Collagenase D	Sigma-Aldrich	Cat. # C5138
Percoll	Sigma-Aldrich	Cat. # C5138
Fixable viability stain	BD Biosciences	Cat. # 565388
Dihydroethidium	Sigma-Aldrich	Cat. # D7008
Hoechst 33342	Sigma-Aldrich	Cat. # B2261
Critical Commercial Assays		
Arcturus PicoPure RNA Isolation Kit	ThermoFisher Scientific	Cat # Kit0204
Nextera XT Kit	Illumina	Cat # FC-131-1-24
DNA High Sensitivity Reagent Kit	PerkinElmer	Cat # CLS760672
Deposited Data		
Raw RNA sequencing data	This paper	GEO115906
Experimental Models: Organisms/Strains		
Mouse: Kit ^{MerCreMer/R26}	Klaus Karjalainen Lab (NTU, Singapore)	Sheng et al., 2015
Mouse: Balb/cJ	The Jackson Laboratory	JAX: 000651
Software and Algorithms		
FeatureCount program	Liao et al., 2014	http://subread.sourceforge.net/
Graphpad Prism v5	Graphpad	https://www.graphpad.com
FlowJo X	TreeStar	https://www.flowjo.com
Rphenograph	Levine et al., 2015	github.com/JinmiaoChenLab/Rphenograph
UMAP	McInnes and Healy, 2018	github.com/lmcinnes/umap

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for reagents may be directed to and will be fulfilled by Lead Contact Christiane Ruedl (ruedl@ntu.edu.sg).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Mice and ethics statement

Kit^{MerCreMer/R26} mice with a genetic (BALB/c x C57BL/6) F1 background were generated by mating *c-kit*^{MerCreMer} mice (BALB/c) with Rosa26^{LSL-eYFP} (C57BL/6) as previously described in ([Sheng et al., 2015](#)). Kit^{MerCreMer/R26} mice were maintained together

with wild-type (WT) BALB/c mice in the animal facility of Nanyang Technological University of Singapore. Kit^{MerCreMer/R26} males were administered tamoxifen injections at 8–10 weeks of age. The study was carried out in strict accordance with the recommendations of the NACLAR (National Advisory Committee for Laboratory Animal Research) guidelines under the Animal & Birds (Care and Use of Animals for Scientific Purposes) Rules of Singapore. The Institutional Animal Care and Use Committee (IACUC) of the Nanyang Technological University of Singapore (Authorization No IACUC SBS/NIE A-0127 and A-0258) approved the protocol.

METHOD DETAILS

Parasite strain, infection protocol and disease assessment

Green fluorescence protein (GFP)-expressing or luciferase-tagged *P. yoelii* 17X clone 1.1, non-lethal strain (Py1.1-GFP or Py17XNL-Luc, respectively) were used for infection. Male mice were injected i.p. with 10⁶ iRBCs. Parasitemia and animal survival were monitored over a period of 35 days by flow cytometry for GFP-expressing parasites or by counting Giemsa-stained parasites in a thin blood smear. Parasite sequestration in organs was determined by bioluminescence as previously described (Gupta et al., 2016).

Tamoxifen-inducible fate-mapping mouse model in Kit^{MerCreMer/R26} mice

Kit^{MerCreMer/R26} mice were used for cell fate-mapping to determine the ontogeny of tissue-resident and immigrant M Φ populations over the course of disease. Tamoxifen (Sigma, T5648) was dissolved in corn oil (Sigma, C8267). Tamoxifen (4 mg/mouse) was administered for five consecutive days by gavage for adult labeling. After tamoxifen administration, c-kit⁺ BM cells in Kit^{MerCreMer/R26} mice become labeled (YFP⁺) and all cells subsequently derived from YFP⁺ BM cells maintain YFP expression. It is therefore possible to distinguish between tissue-resident M Φ of either embryonic origin or those derived from adult BM definitive hematopoiesis following *Plasmodium* infection.

Cell isolation and flow cytometry

Cells were isolated as described in (Sheng et al., 2015). Briefly, after CO₂ euthanasia of mice, liver, spleen, and lungs were removed, cut into small pieces, and digested in IMDM containing 2% FCS and 1 mg/ml Collagenase D (Sigma) under shaking conditions at 37°C for 1 h. Digested pieces were gently mashed through a cell strainer. The leukocyte population was enriched by a 35% Percoll gradient (Sigma), and the cell pellet was harvested and further processed for flow cytometry staining.

Liver, spleen, and lung single-cell suspensions were first incubated with 10 μ g/ml Fc block (Clone 2.4G2) and fixable viability stain (BD Biosciences), then subsequently stained and analyzed on a BD Fortessa 5-laser flow cytometer. Ki-67 intracellular staining was performed according to the manufacturer's instructions (eBioscience, San Diego, CA, USA). The following antibodies from BioLegend (San Diego, CA, USA) were used: PE-Cy7-labeled CD11c (clone: N418), APCFire-labeled CD45 (clone: 30F11), BV605-labeled Ly6C (HK1.4), FITC-labeled Ly6G (clone: 1A8), PE-labeled EMR1 (also known as F4/80) (clone: BM8), BV421-labeled MHCII (clone: M5/114.15.2). BUV395-labeled CD11b (clone: M1/70) was obtained from BD Bioscience. Ki67 (clone: SolA15), eFluor660 was obtained from eBioscience. Data were analyzed using FlowJo X software (TreeStar, Ashland, OR, USA).

Transcriptomics analysis by RNA-sequencing and bioinformatics analysis

Tissue-resident M Φ obtained from the liver, lungs, and spleen of 8–10 uninfected or *P. yoelii*-infected BALB/c mice at 35 d.p.i. were purified by cell sorting using a BD FACSAria cell sorter. Total RNA was extracted using the Arcturus PicoPure RNA Isolation Kit (Thermo Fisher Scientific) according to the manufacturer's protocol. All mouse RNAs were analyzed on an Agilent Bioanalyzer for quality assessment; RNA Integrity Number (RIN) range was 6.5–10, median of 9.2. cDNA libraries were prepared with 2 ng of total RNA and 1 μ l of a 1:50,000 dilution of ERCC RNA Spike in Controls (Ambion) using the SMARTSeq v2 protocol (Picelli et al., 2014) using 20 μ M TSO and 250 pg of cDNA with 1/5 reaction of Illumina Nextera XT kit (FC-131-1-24). The length distribution of the cDNA libraries was monitored using DNA High Sensitivity Reagent Kit (CLS760672) on the Perkin Elmer Labchip. All eight samples were subjected to an indexed PE sequencing run of 2x51 cycles on an Illumina HiSeq 2500 Rapid mode (17 samples/lane).

The paired-end reads were mapped to the Mouse GRCm38/mm10 reference genome using the STAR alignment tool (Dobin et al., 2013). Mapped reads were summarized to gene level using featureCounts (V1.5.0-P1) software (Liao et al., 2014) and with GENCODE gene annotation, Release M10 (Harrow et al., 2006). Genes with average number of reads per sample less than 10 in all cell subpopulations were filtered out from further analysis.

QUANTIFICATION AND STATISTICAL ANALYSIS

Cell subpopulations analysis

Data were analyzed using Prism 5 (GraphPad Software). Statistical details, including statistical tests used, number of mice analyzed (n) can be found in the legend for each figure. All data are presented as the mean \pm standard error of the mean (SEM). Statistical comparisons among two groups were determined by nonparametric two-tailed Mann-Whitney U test and was defined as follows: *, p < 0.05; **, p < 0.01; ***, p < 0.001; ****, p < 0.0001; ns, non-significant.

High-dimensional flow cytometry data analysis

For Phenograph cell clustering and UMAP dimensionality reduction, custom R and Python scripts were used. After manually gating on cells of interest and exporting using FlowJo, .fcs files were opened in R using the flowCore (Bioconductor.org) package. Light scattering parameters “FSC-A” and “SSC-A” were normalized (minimum = 0, maximum = 4.5) on a linear scale. Fluorescent parameters were Log-transformed (using flowCore functions) with automatically determined “w” and “t” parameters, and “m” parameter set to 4.5. After transformation, PhenoGraph clustering was performed using the Rphenograph package (github.com/JinmiaoChenLab/Rphenograph), $k = 20$. For UMAP, transformed values were exported and used for input for a custom python script for implementing the “umap” Python package (([McInnes and Healy, 2018](https://doi.org/10.1038/nature24046)) github.com/lmcinnes/umap), using parameters: $n_neighbors = 15$, $min_dist = 0.2$, $metric = "euclidean"$, $n_components = 1$. Calculated values were then plotted in R as dot-plots colored by relative marker intensities or as heatplots (using the `heatplot.2` function and default parameters for hierarchical clustering) of median marker intensities within each PhenoGraph cluster. These scripts will be made available upon reasonable request.

RNaseq analysis

For differentially expressed gene (DEG) analysis, one of the best performing RNA-seq data analysis pipelines recommended by the MicroArray Quality Control (MAQC) project ([SEQC/MAQC-III Consortium, 2014](https://doi.org/10.1093/bioinformatics/btu087)), limma/voom pipeline was used. Comparisons of the pre- and post-infection M Φ for each of the tissues were done using limma and DEGs were selected with Benjamini-Hochberg multiple testing correction adjusted p values of < 0.05 . Hierarchical clustering and principal component analysis were done with Log₂-transformed value of Fragments Per Kilobase of transcript per Million mapped reads (FPKM). All RNA-Seq analyses were performed in R-3.1.2 (URL <http://www.R-project.org/>).

DATA AND SOFTWARE AVAILABILITY

All expression data related to this manuscript can be found at Gene Expression Omnibus under the accession number GEO115906. The FeatureCount program is accessible at <http://subread.sourceforge.net/>.

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Supplemental Information

**Organ-Specific Fate, Recruitment,
and Refilling Dynamics of Tissue-Resident
Macrophages during Blood-Stage Malaria**

Si Min Lai, Jianpeng Sheng, Pravesh Gupta, Laurent Renia, Kaibo Duan, Francesca Zolezzi, Klaus Karjalainen, Evan W. Newell, and Christiane Ruedl

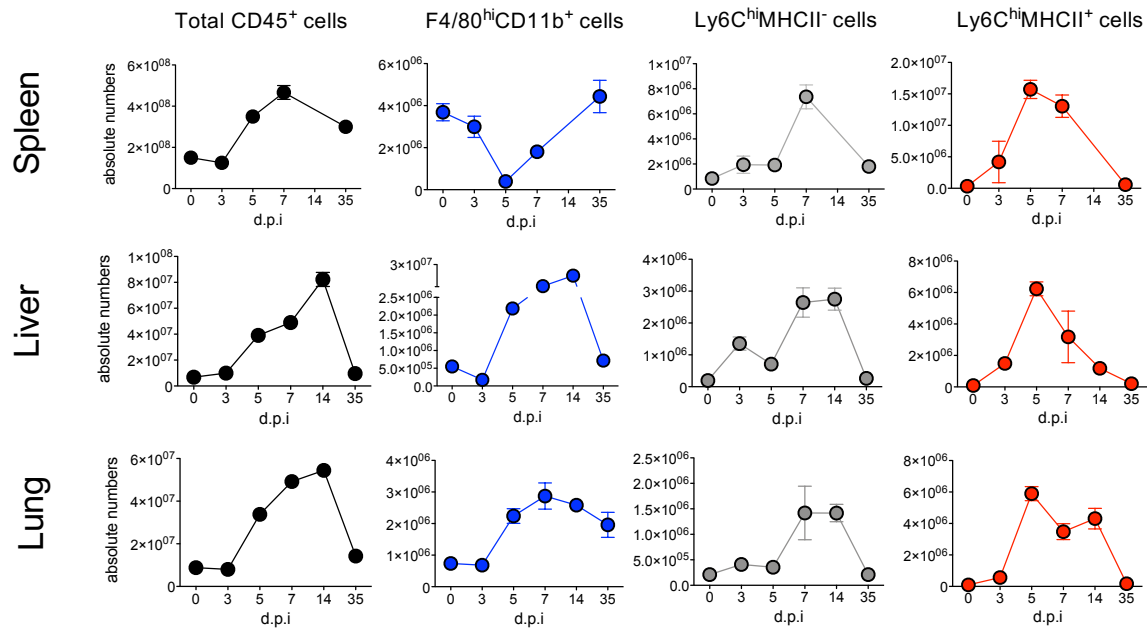


Fig. S1 (related to Fig. 2): Graphical representation of the absolute numbers of total cells (black curve), of F4/80^{hi} macrophages (blue curve), Ly6C⁺MHC II⁻ (grey curve) and Ly6C⁺MHC II⁺ monocytes (red curve) during the time course of *P. yoelii* blood-stage infection. Each value represents the percentage of the correspondent cell population +/- SEM (3–4 mice/group and time point).

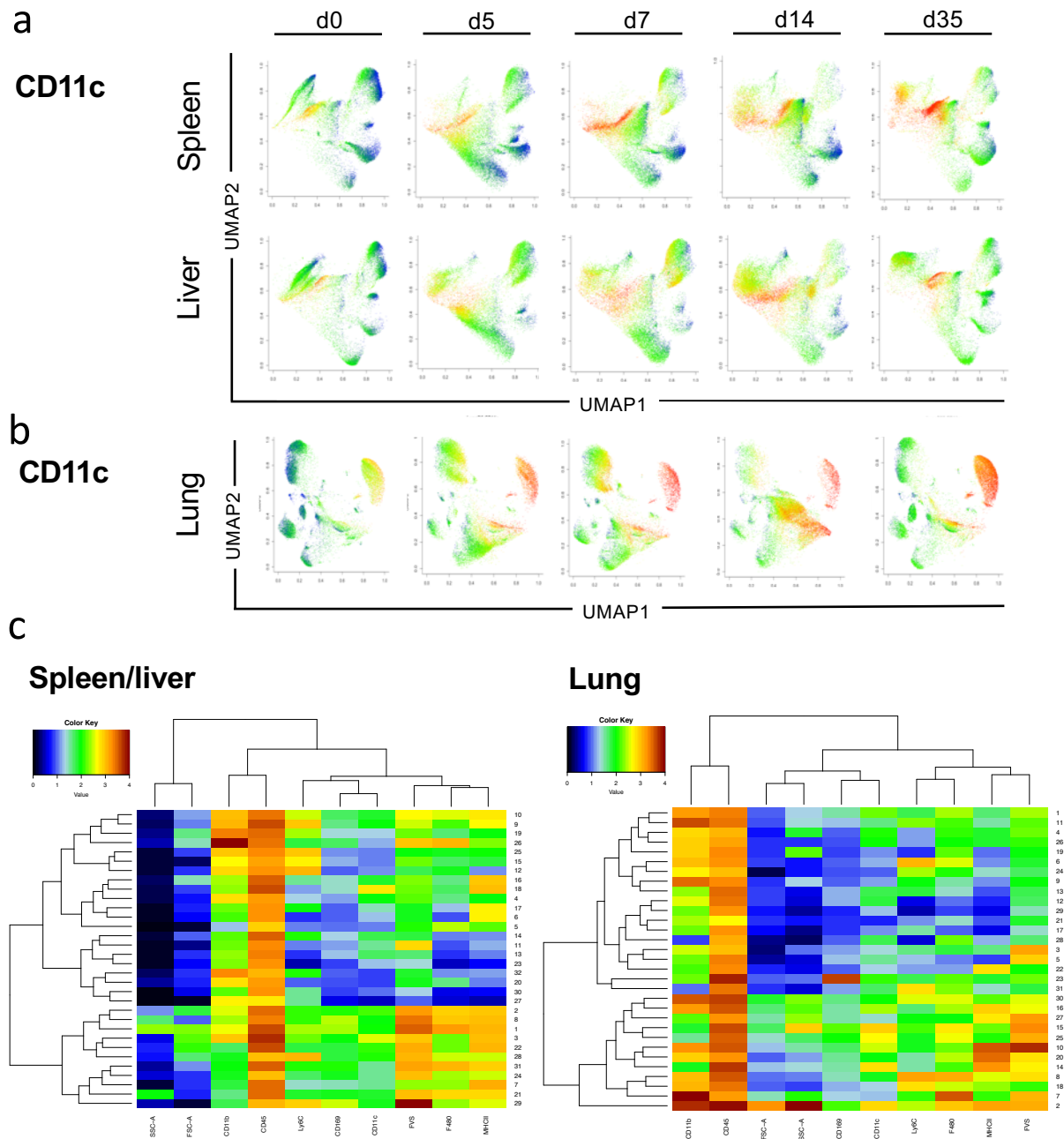


Fig. S2 (related to Fig. 4): Expression plots of CD11c color mapped from blue (low expression) to red (high expression) in (a) spleen (upper), liver (bottom) and (b) lungs during different stages of *P. yoelii* infection. (c) Heat maps of median parameter (columns) expression values (post-transformation values, see methods) for each Phenograph cluster (rows) for the concatenated data obtained from spleen and liver (left) and the separately-analyzed lung samples (right)

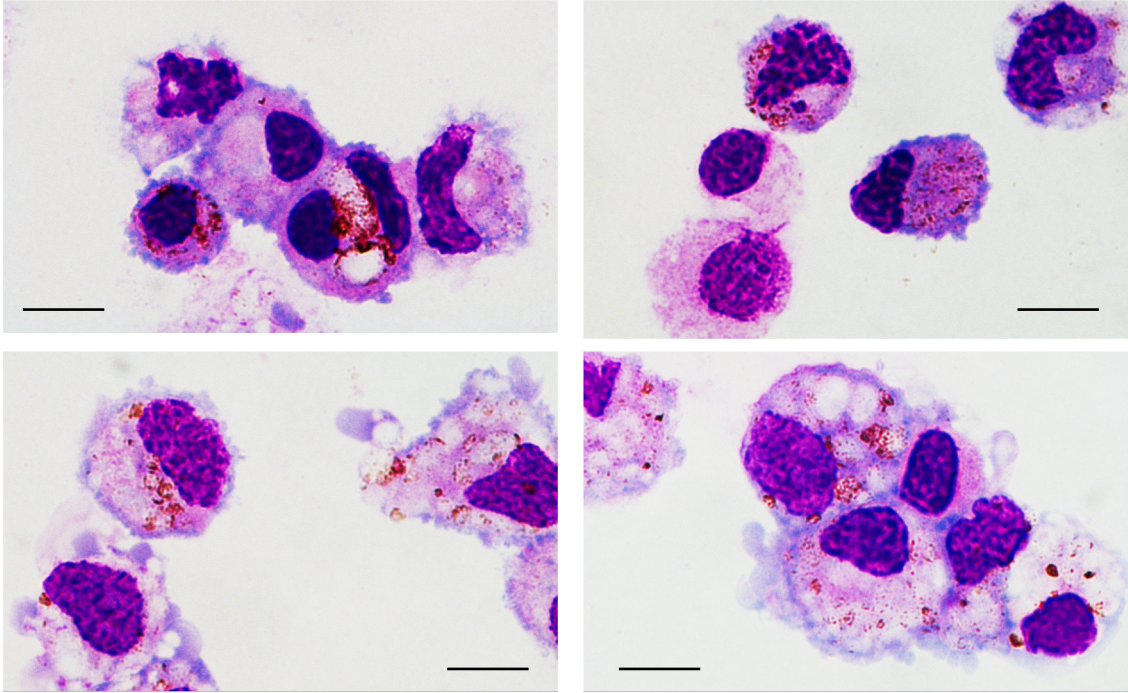


Fig. S3 (related to Fig. 4): Cytopspins of F4/80^{hi} Kupffer cells sorted from *P. yoelii* infected livers. Visible brown inclusions are intracytoplasmic haemazoin pigments. Scale bar: 10 μ m

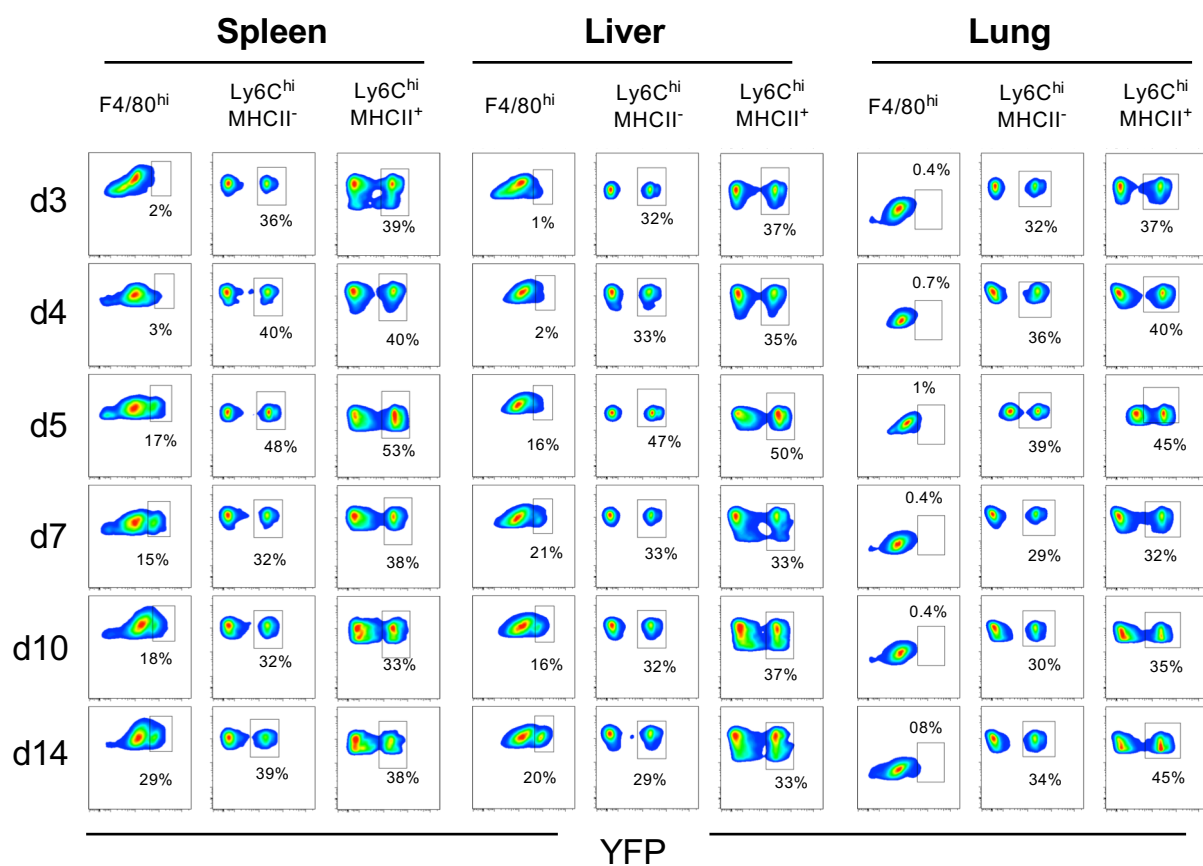


Fig. S4 (related to Fig. 5): Representative flow cytometry analysis indicating the labelling efficiency of distinct myeloid cell populations in spleen, liver and lungs during *P. yoelii* infection: F4/80^{hi} tissue-resident macrophages; Ly6C^{hi}MHCII⁻ monocytes and Ly6C^{hi}MHCII⁺ monocytes-derived cells. Representative dot plots (3, 4, 7, 10, 14 days post infection) showing YFP labelling percentage.

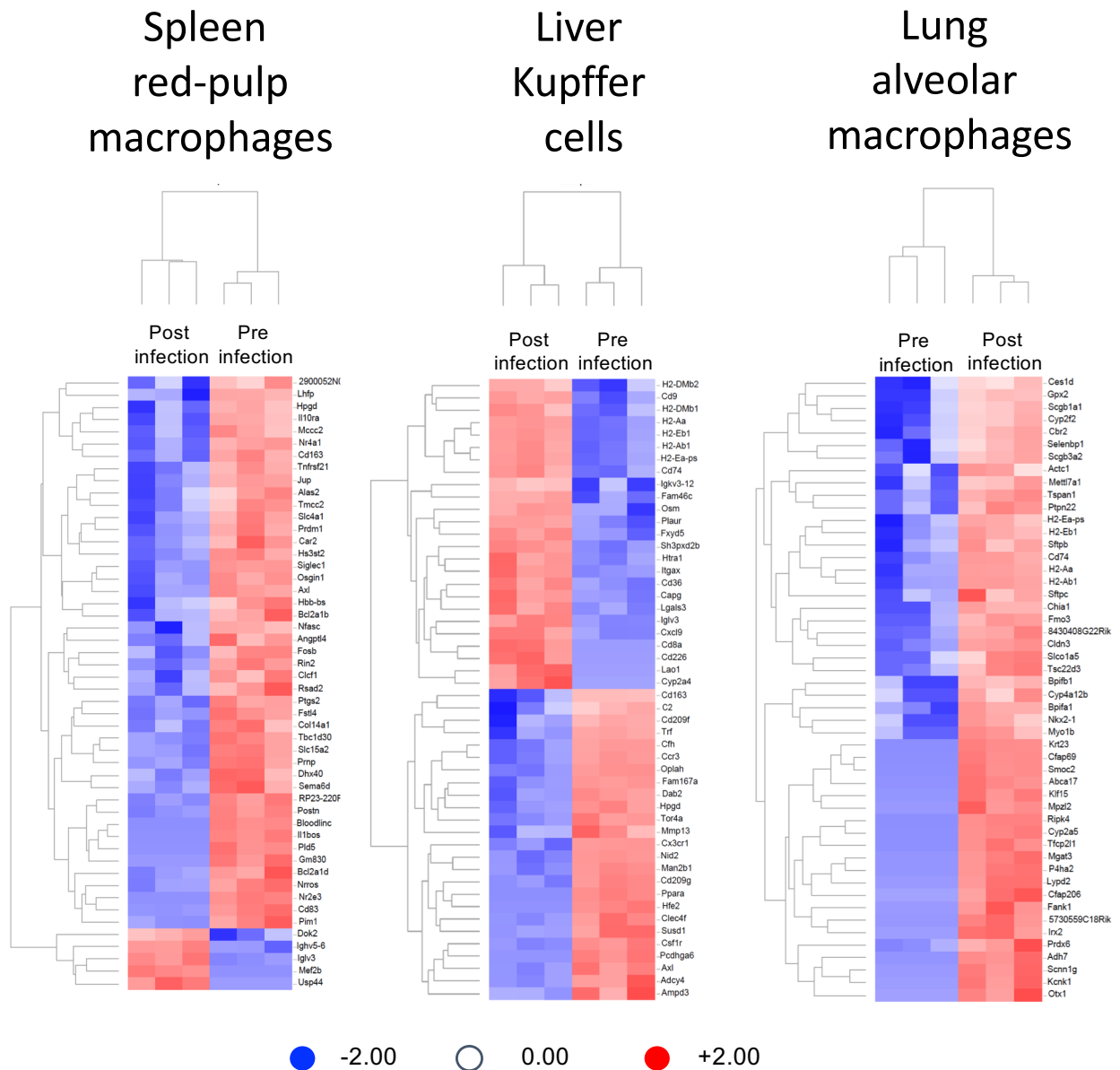
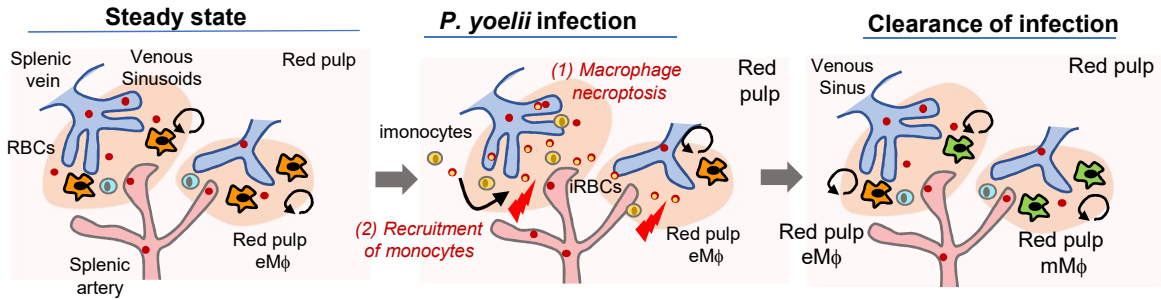
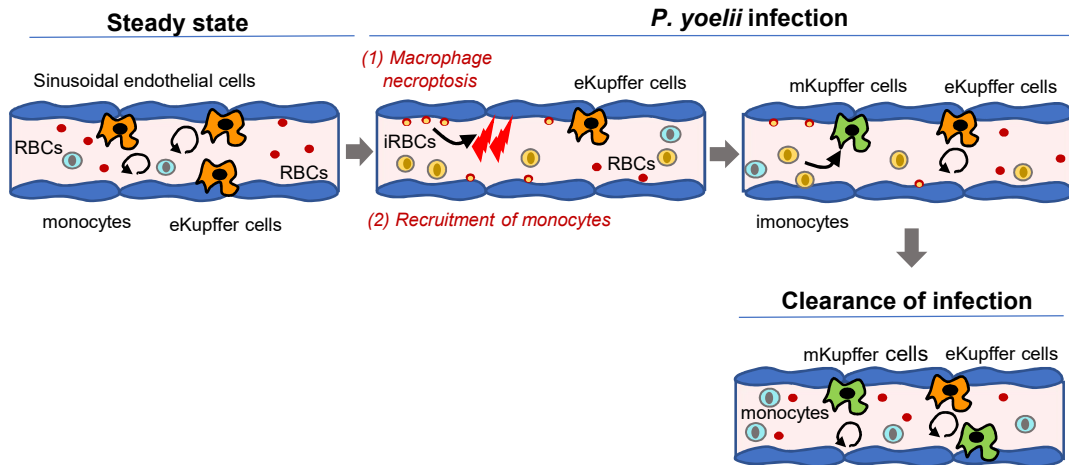


Fig. S5 (related to Fig. 6): Heatmaps for DEGs of spleen, liver and lung macrophages, respectively (pre infection versus post infection). The top 50 DEGs based on the adjusted p-values are shown. Log₂ fold change in gene expression is denoted by negative -2 (blue) to positive +2 (red).

Spleen



Liver



Lungs

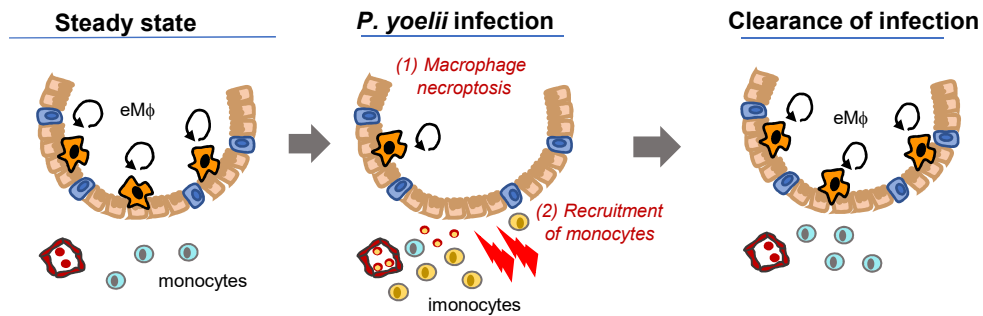


Fig. S6 (related to Fig. 5 and 7): Schematic representation of macrophage replenishment dynamics in spleen, liver and lungs during *P. yoelii* blood-stage malaria.