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Chronic TLR7 and TLR9 signaling drives anemia via differentiation of specialized hemophagocytes

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Abstract

Cytopenias are an important clinical problem associated with inflammatory disease and infection. We show that specialized phagocytes that internalize red blood cells develop in TLR7-driven inflammation. TLR7 signaling caused the development of inflammatory hemophagocytes (iHPC), which resemble splenic red pulp macrophages, but are a distinct population derived from Ly6C^{hi} monocytes. iHPCs were responsible for anemia and thrombocytopenia in TLR7-overexpressing mice, which have a macrophage activation syndrome (MAS)-like disease. IRF5, associated with MAS, participated in TLR7-driven iHPC differentiation. We also found iHPCs during experimental malarial anemia, where they required endosomal TLR and MyD88 signaling for differentiation. Our findings uncover a mechanism by which TLR7 and TLR9 specify monocyte fate, and identify a unique population of phagocytes responsible for anemia and thrombocytopenia associated with inflammation and infection.

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Data and Materials availability: RNA-Sequencing data are deposited in the NCBI Gene Expression Omnibus under SuperSeries GSE117718 containing CMP dataset (GSE70520) and iHPC dataset (GSE117711). All other data needed to evaluate the conclusions in this paper are present either in the main text or the Supplementary Materials.

SUPPLEMENTARY MATERIALS

Materials and Methods

Figs. S1 to S11

One Sentence Summary:

Specialized phagocytes required for inflammation-induced cytopenias differentiate in response to TLR7/9 signaling.

Inflammatory disorders and infections are associated with cytopenias, including anemia and thrombocytopenia. However, the mechanisms underlying these phenomena are poorly understood. A particularly acute and life-threatening form of inflammatory cytopenia is seen in macrophage activation syndrome (MAS), in which activated macrophages containing red blood cells (RBCs) and leukocytes are found in the bone marrow, spleen, and liver (1, 2). MAS is a severe complication of some rheumatological diseases, most commonly in systemic juvenile idiopathic arthritis (sJIA) and pediatric lupus, or can develop after viral infections, such as with Epstein–Barr virus (EBV) (1, 3, 4). Severe cytopenias, principally anemia and thrombocytopenia, also occur during acute malaria infection, particularly in young children, where it can cause mortality (5–7). Thus, an understanding of the mechanisms underlying cytopenias that accompany inflammatory disease and infection is important in order to identify points of therapeutic intervention for these conditions.

The phagocytosis of RBCs, platelets, and leukocytes can be a major contributor to acute cytopenias. Thus, we reasoned that specialized phagocytes may develop in inflammatory conditions in response to the signaling of pattern recognition receptors, such as Toll-like receptors (TLRs). TLRs are well known to trigger cytokine production from mature myeloid cells, such as macrophages. However, little is known about the role of TLRs in specifying myeloid cell development. TLRs are highly expressed on hematopoietic stem and progenitor cells (HSPCs) beginning with hematopoietic stem cells (HSCs) through to more committed myeloid progenitor cells, such as common myeloid progenitors (CMPs). The treatment of HSPCs with TLR agonists in vitro can induce macrophage differentiation in the absence of the homeostatic macrophage differentiation factor M-CSF. However, it is unclear whether this occurs in vivo (8–10). Viral or bacterial infection or injection of mice with TLR agonists lead to characteristic changes in HSPCs, which correlate with the increased production of neutrophils and monocytes in a process termed “emergency myelopoiesis” or “demand-adapted hematopoiesis” (11). However, many of these effects depend upon the TLR-induced production of inflammatory cytokines, including IL-3, IL-6, IFN γ , and type I IFN, from mature myeloid cells or non-hematopoietic cells that promote myelopoiesis (10, 12). Thus, in contrast to its potent effects in vitro, the cell-intrinsic role of TLR signaling in directing monocyte/macrophage differentiation in vivo remains an open question.

TLR7 promotes hemophagocyte development in vitro and in vivo

We hypothesized that TLR signaling may drive a specialized macrophage phenotype. To test this hypothesis, we used an in vitro culture system in which bone marrow common myeloid progenitors (CMPs) were cultured with the TLR7 agonist R848. This approach efficiently induced the differentiation of CD11b⁺F4/80⁺ macrophages after 4 days (Fig. 1A) (9). We compared the transcriptional profiles of macrophages differentiated from CMPs with R848 to those differentiated with the homeostatic macrophage-differentiating growth factor M-CSF, and found 813 upregulated and 1,020 downregulated transcripts (2-fold, FDR 0.05)

in macrophages from R848 compared to M-CSF cultures (Fig. 1B). Interestingly, the gene encoding the transcription factor Spi-C (*SpiC*), which governs splenic red pulp macrophage (RPM) development (13) was significantly upregulated, suggesting TLR7 signaling in CMPs may preferentially promote differentiation of RPM-like cells (Fig. 1B). These R848-differentiated macrophages most strongly resembled RPMs in comparison to microglia, peritoneal macrophages, and alveolar macrophages (Fig. 1C). Furthermore, they were enriched for a core set of genes that distinguish RPMs from these other tissue macrophage populations (14) (Fig. 1D). The hallmark function of RPMs is the phagocytosis of RBCs (“hemophagocytosis”) (15). Indeed, R848-differentiated macrophages efficiently phagocytosed RBCs (Fig. 1E). Thus, TLR7 signaling in CMPs dictates the development of hemophagocytic RPM-like macrophages in vitro.

To examine if TLR7 signaling increased hemophagocyte development in vivo, we used TLR7.1 mice (16), which overexpress TLR7 approximately tenfold under its own regulatory elements, including in HSPCs and mature myeloid cells (12). Among CD45⁺ leukocytes that had internalized RBCs (as determined by intracellular anti-Ter-119 staining), we identified a small number of hemophagocytic leukocytes in the spleens of wild-type (WT) mice, which exhibited cell surface staining (CD11b^{int/lo}F4/80^{hi}) consistent with RPMs (Fig. 2A-C). Significantly higher numbers of hemophagocytes were found in the spleen and bone marrow of TLR7.1 mice (Fig. 2A and B, fig. S1). These cells had increased levels of intracellular anti-Ter-119 staining, suggesting that hemophagocytes in TLR7.1 mice were more phagocytic than in WT mice. Furthermore, although a small population of hemophagocytes in TLR7.1 mice resembled RPMs by F4/80 and CD11b staining, the majority were CD11b^{int/hi}F4/80^{lo} (Fig. 2C). These hemophagocytes lacked the high VCAM and CD64 expression associated with RPMs and expressed higher levels of CD31 and CD55. They also expressed similar levels of SIRPα and TREML4 compared to RPMs (Fig. 2D). Thus, most hemophagocytes in TLR7.1 mice appeared to represent a population of hemophagocytes distinct from RPMs. Hemophagocytes with the identical phenotype were found in the spleen after daily injection with the TLR7 agonist R848 (Fig. S2). As these CD11b^{int/hi}F4/80^{lo} hemophagocytes did not exist in untreated or non-inflamed WT mice, we have termed them “inflammatory hemophagocytes” or iHPCs.

Gating iHPCs and RPMs using only cell surface markers (Fig. S3) showed that TLR7.1 iHPCs were approximately two-to-threefold more phagocytic than RPMs from either WT or TLR7.1 mice as measured by intracellular anti-Ter-119 staining (Fig. 2E). Sorting of iHPCs and RPMs followed by hematoxylin and eosin staining confirmed that the anti-Ter-119 staining corresponded to internalized RBCs, and showed that more iHPCs contained multiple RBCs, confirming these cells were more phagocytic than RPMs (Fig. 2F and 2G). Thus, iHPCs, though similar to RPMs, are a distinct population of hemophagocytes.

iHPCs differentiate in response to cell-intrinsic TLR7 signals and do not require Spi-C

We next investigated the developmental pathway of iHPCs. To determine if iHPCs require cell-intrinsic TLR7 signaling to develop or if the inflammation present in TLR7.1 mice is

sufficient for this process, we used mixed bone marrow chimeras. Whereas splenic B cells and monocytes reconstituted at the 1:1 ratio of the input bone marrow, iHPCs were almost uniquely derived from TLR7.1 bone marrow with an ~100:1 ratio of TLR7.1:WT iHPCs (Fig. 3A, fig. S4A-C). Thus, iHPCs uniquely develop in response to cell-intrinsic chronic TLR7 signaling.

We used the R848 injection model to determine whether TLR7 signaling is required for iHPC development. After R848 injection in mixed WT: *Tlr7*^{-/-} bone marrow chimeras, monocytes were found at the 1:1 WT: *Tlr7*^{-/-} ratio of the input bone marrow. However, iHPCs were heavily skewed towards WT cells at a ratio of ~100:1 (Fig. 3B, S5A and B). Thus, cell-intrinsic TLR7 signaling promotes, and is required for, iHPC development in vivo. Therefore, TLR7 signaling plays a critical role in the induction of a specific macrophage phenotype induced during TLR7-driven inflammation.

The transcription factor Spi-C is required for RPM development (13) and is also highly expressed in macrophages generated by TLR7 signaling in vitro (Fig. 1B, D). Splenic iHPCs sorted directly ex vivo from TLR7.1 mice also expressed high levels of *Spic* mRNA comparable to RPMs, and in contrast to splenic Ly6C^{hi} monocytes (Fig. S6A). Because of the functional similarities between RPMs and iHPCs and our observation that both express high levels of Spi-C, we hypothesized this transcription factor would be required for iHPC differentiation. Therefore, we generated TLR7.1/*Spic*^{-/-} mice. As expected, both *Spic*^{-/-} and TLR7.1/*Spic*^{-/-} mice had few splenic RPMs, and *Spic*^{-/-} mice showed a significant reduction in total hemophagocytes in comparison with WT mice (Fig. S6B-D), as most of these cells are *Spic*-dependent RPMs. Surprisingly, TLR7.1/*Spic*^{-/-} mice had similar numbers of splenic hemophagocytes as TLR7.1/*Spic*^{+/+} and TLR7.1/*Spic*^{+/-} mice (Fig. S6C). When the total hemophagocyte population was separated into RPMs and iHPCs (Fig. S6D and E), there was no difference in iHPC frequency or number in TLR7.1 mice regardless of *Spic* expression. Similarly, in our in vitro differentiation system, there was no difference in R848-induced macrophage differentiation from *Spic*^{+/+} and *Spic*^{-/-} CMPs (Fig. S6F). Thus, Spi-C is not required for iHPC development in vivo or in vitro, demonstrating that iHPCs develop through a program distinct from RPMs.

iHPCs differentiate from Ly6C^{hi} monocytes

In the steady state, many tissue macrophages, including RPMs, are derived from embryonic yolk sac- or fetal liver-derived progenitors that locally self-renew. However, monocytes can replenish some, but not all, of these populations both in the steady state or during inflammation or other perturbations (17–19). For example, RPMs can be replaced by “classical” or “inflammatory” Ly6C^{hi} monocyte-derived cells after RPM death due to heme-mediated toxicity (20). Thus, we asked whether TLR7-induced iHPCs are derived from Ly6C^{hi} inflammatory monocytes. iHPCs did not express the defining inflammatory monocyte markers CCR2 or Ly6C, and had low expression of the tissue macrophage marker CD64 similar to Ly6C^{hi} monocytes (Fig. 4A). Ly6C^{hi} monocytes also expressed low levels of the iHPC marker CD31. Culture of Ly6C^{hi} monocytes with TLR7 ligands induced phenotypic changes associated with iHPCs, including the induction of *Spic* and *Pecam1* (encoding CD31), and the reduced expression of *Ccr2* and *Ly6c1* (Fig. 4B). Thus, although

Ly6C^{hi} monocytes had a distinct cell surface phenotype from iHPCs, TLR7 signaling in Ly6C^{hi} monocytes caused transcriptional changes associated with the iHPC phenotype, suggesting that iHPCs differentiate from Ly6C^{hi} monocytes in response to TLR7 signals.

To directly assess whether iHPCs derive from Ly6C^{hi} monocytes, we crossed TLR7.1 mice to *Ccr2*-DTR mice, in which injection of diphtheria toxin (DT) results in the depletion of Ly6C^{hi} monocytes within 24 hours and which can be maintained over time with subsequent DT injections (21–23). Ly6C^{hi} monocyte depletion over 2.5 weeks (Fig. S7A and B) caused a significant reduction in total splenic hemophagocytes and iHPCs in TLR7.1/*Ccr2*-DTR mice, in comparison with TLR7.1 mice not expressing the *Ccr2*-DTR transgene (Fig. 4C, D). Consistent with the fact that iHPCs do not express CCR2 (Fig. 4A), a single DT injection into TLR7.1/*Ccr2*-DTR mice depleted Ly6C^{hi} monocytes, but not iHPCs, after 24 hours (Fig. S7B, C). Thus, iHPCs were not directly depleted by DT injections. Interestingly, 2.5 weeks of DT treatment also significantly reduced RPM numbers in TLR7.1/*Ccr2*-DTR mice (Fig. S7E), suggesting that during chronic TLR7-driven inflammation, not only are iHPCs derived from Ly6C^{hi} monocytes, but RPMs are also replaced by monocyte-derived cells.

The transcriptional changes in Ly6C^{hi} monocytes associated with the iHPC phenotype were not unique to TLR7 signaling. Signaling via TLR9 through CpG DNA also induced *Spic* and *Pecam1*, and reduced *Ccr2* and *Ly6c1* in Ly6C^{hi} monocytes (Fig. 4E). Notably, signaling via TLR4 only caused some of the iHPC transcriptional changes—LPS induced *Spic* and reduced *Ccr2*, but did not induce *Pecam1* or reduce *Ly6c1* expression (Fig. 4E). We also assessed IL-1 β , which, similar to TLRs, signals via MyD88. IL-1 β signaling was similar to LPS, in that it induced *Spic*, but not *Pecam1* (Fig. S8A). We also tested signaling with poly(I:C) through TLR3, which uses TRIF exclusively and is expressed at low levels on Ly6C^{hi} monocytes (14). TLR3 signaling did not significantly induce *Spic* and *Pecam1* or reduce *Ly6c1* expression. However TLR3 signaling did significantly reduce *Ccr2* expression to a lesser extent than other TLR agonists tested (Fig. S8B). Finally, IFN γ , a classic monocyte activator, did not induce any of the transcriptional changes associated with iHPC differentiation in Ly6C^{hi} monocytes (Fig. S8A). Thus, TLR7 and TLR9 signaling uniquely drive the iHPC phenotype in Ly6C^{hi} monocytes.

To further assess how iHPCs are related to Ly6C^{hi} monocytes and RPMs, we performed RNA-Seq analysis of these populations from naïve WT and TLR7.1 mice. Principal component analysis of these data revealed that TLR7.1 iHPCs cluster separately from both Ly6C^{hi} monocytes and RPMs, whether they were derived from WT or TLR7.1 mice (Fig. 4F), reinforcing their unique iHPC identity. Unsupervised hierarchical clustering of iHPCs, Ly6C^{hi} monocytes, and RPMs from TLR7.1 mice using significantly differentially expressed genes (FC>1.5, FDR<0.05) between these three populations also highlighted these differences, and demonstrated a closer relationship between iHPCs and Ly6C^{hi} monocytes than iHPCs and RPMs. We also used this dataset to identify unique surface markers of iHPCs to better distinguish them from Ly6C^{hi} monocytes and RPMs by flow cytometry (Fig. S9A). Of those tested, DR3, encoded by *Tnfrsf25*, was best at identifying iHPCs as it is expressed in neither Ly6C^{hi} monocytes nor RPMs. DR3 used in conjunction with CD31 reliably identified TLR7.1 splenic iHPCs that were uniformly expressing Spi-C and enriched

for hemophagocytes (Fig. S9B-D). Thus, transcriptional profiling supports our contention that iHPCs are a unique population of Ly6C^{hi} monocyte-derived cells.

Monocyte-derived iHPCs drive TLR7 and TLR9-induced MAS-like disease

The presence of iHPCs in TLR7.1 mice was associated with anemia characterized by reduced RBC count that typically developed by 3 months of age (Fig. 5A) (16). Indeed, the percent and number of splenic iHPCs was inversely correlated with the RBC count in a cohort of TLR7.1 mice (Fig. 5B). The lack of RPMs, but not iHPCs, in TLR7.1/*Spic*^{-/-} mice allowed us to assess the contribution of RPMs versus iHPCs in anemia. Similar to published results, we found no difference in RBC count between *Spic*^{+/+}, *Spic*^{+/-}, and *Spic*^{-/-} mice, suggesting that in the steady state Spi-C-dependent RPMs are not major contributors to RBC homeostasis (13). TLR7.1/*Spic*^{-/-} mice developed anemia similarly to TLR7.1/*Spic*^{+/+} and TLR7.1/*Spic*^{+/-} mice (Fig. 5C) showing that RPMs do not contribute to TLR7-driven anemia, and suggesting that iHPCs are sufficient to cause this outcome.

The lack of iHPCs in DT-injected TLR7.1/*Ccr2*-DTR mice allowed us to ask if iHPCs drive anemia in the TLR7.1 model by beginning depletions once anemia was detected (Fig. S7A). TLR7.1 mice not expressing the *Ccr2*-DTR transgene became progressively more anemic during the course of DT treatment, whereas anemia was completely reversed in the DT-treated TLR7.1/*Ccr2*-DTR mice (Fig. 5D). Taken together with the sustained anemia in TLR7.1/*Spic*^{-/-} mice lacking RPMs, we conclude that hemophagocytosis by iHPCs is required for TLR7-driven anemia. We also investigated a model of anemia caused by chronic TLR9 signaling induced with repetitive CpG DNA injection in *Ccr2*-DTR mice (21). Similar effects of monocyte and iHPC depletion were observed in this TLR9-dependent model (Fig. 5E), indicating that hemophagocytosis by iHPCs may be a common mechanism of anemia associated with TLR7 and/or TLR9-dependent inflammation.

The anemia in TLR7.1 mice is reminiscent of the spectrum of diseases defined as MAS, in which activated macrophages cause severe cytopenias by phagocytosing RBCs as well as leukocytes and platelets (1, 2). We confirmed that TLR7.1 mice have very low platelet counts (16), even when the RBC count is still close to the range of wild-type mice (Fig. 5F). Similar to RBC counts, platelet counts were completely restored after 2.5 weeks of Ly6C^{hi} monocyte depletion in TLR7.1/*Ccr2*-DTR mice (Fig. 5F). Thus, iHPCs contribute to both anemia and thrombocytopenia, and likely other aspects of the MAS-like disease in TLR7.1 mice.

This led us to ask which receptors might allow iHPCs to phagocytose RBC, and potentially platelets and other cells. Analysis of receptors, bridging molecules' and transcription factors that have been implicated in phagocytosis of self cells in our RNA-Seq dataset showed that there is a subset of these molecules expressed more highly in iHPCs than monocytes and RPMs, including genes encoding the α v integrin chain, CD300f, and the β 2 integrin chain (22) (Fig. S10). Additionally, genes for hemophagocytosis may be shared with RPMs, indicating possible roles for C1q, Mer (encoded by *Mertk*), and LXR α (encoded by *Nr1h3*), which are all more highly expressed in iHPCs and RPMs than monocytes.

IRF5 participates in iHPC differentiation

We propose that cytopenias in MAS can be caused by direct chronic endosomal TLR signaling in monocytes or myeloid progenitor cells. Whether due to infection, genetic perturbations in TLR signaling, and/or the increased availability of nucleic acid endosomal TLR ligands, this may directly lead to the differentiation of pathogenic iHPCs. Indeed, some forms of MAS are associated with variants in the gene encoding the IRF5, a transcription factor activated downstream of TLR signaling in monocytes and macrophages (23, 24). The MAS-associated SNPs in *IRF5* are proposed to increase TLR signals (25–27). Accordingly, we investigated whether IRF5 is involved in TLR7-induced iHPC differentiation. Induction of *Spic* and *Pecam1* was significantly reduced in IRF5-deficient Ly6C^{hi} monocytes compared with WT monocytes, whereas downregulation of *Ccr2* and *Ly6c1* did not depend on IRF5 (Fig. 6A). The in vivo induction of iHPCs after R848 injection was significantly reduced in *Irf5*^{-/-} mice compared to WT mice (Fig. 6B, C). Thus, IRF5 is required for optimal iHPC differentiation and increased IRF5 signaling is genetically associated with MAS.

iHPCs differentiate in response to endosomal TLR signaling in a pre-clinical model of severe malarial anemia

In addition to MAS, severe cytopenias accompany malaria caused by infection with *Plasmodium falciparum* and *P. vivax*, particularly the anemia and thrombocytopenia seen in severe malarial anemia (5–7). Reasoning that iHPCs may also develop during malaria, we infected mice with *Plasmodium yoelii* 17XNL-infected RBCs, a pre-clinical, non-lethal model of blood stage malaria, which causes severe anemia and thrombocytopenia (Fig. 7A). Indeed, RBC-stage *P. yoelii* infection induced hemophagocytosis (Fig. 7B, C) (7). iHPCs were seen as early as day 5 after infection and iHPC number peaked at the time of most severe anemia and correlated inversely with RBC count (Fig. 7D, E). We also found iHPC differentiation during MCMV infection of young Balb/c mice in a model of virally-induced MAS (Fig. S11) (28). Thus, iHPCs also differentiate during infection.

We next investigated whether TLR signals are required for *Plasmodium*-induced iHPC differentiation. *P. yoelii*-infected *Myd88*^{-/-} mice, which lack most TLR signaling as well as IL-1R and IL-18R signaling, showed significantly reduced iHPC differentiation and anemia compared to WT mice (Fig. 7F and G). Notably, *Myd88*^{-/-} mice showed reduced parasitemia than WT mice, which may reflect the increased RBC count in *Myd88*^{-/-} mice or a role for iHPCs in the clearance or sequestration of infected RBC in tissues. Mixed bone marrow chimeras showed a cell-intrinsic requirement for the TLR signaling adapters MyD88 and TRIF and for the endosomal TLR chaperone UNC93B1 in iHPC differentiation during RBC stage *Plasmodium yoelii* 17XNL infection (Fig. 7H, I). Thus, cell-intrinsic endosomal TLR signaling, likely via TLR7 and/or TLR9, also drives iHPC differentiation during experimental malarial anemia.

Discussion

TLR signaling has previously been shown to cause macrophage differentiation in vitro. However, in vivo evidence for a cell-intrinsic role of TLRs in this process and in defining macrophage fate has been lacking. Our work reveals a unique developmental pathway by which myeloid progenitors and Ly6C^{hi} monocytes respond directly to chronic TLR7 and TLR9 signaling in vitro and in vivo by inducing hemophagocytes similar to, yet distinct from, RPMs. We identified iHPCs not only in a transgenic model of TLR7 overexpression resulting in a MAS-like disease, but also during RBC-stage *P. yoelii* 17XNL infection and during infection with MCMV. Thus, iHPCs differentiate in a broad array of situations that have systemic TLR7 and/or TLR9 activation in common (29–37). Not only do these models share innate sensors, but they also share anemia and thrombocytopenia during the course of infection or disease.

The inflammatory hemophagocyte program is unique to TLR7 and TLR9 signaling. In vivo, both chronic TLR7 and TLR9 signaling drive anemia, and we have precisely assessed the contribution of TLR7 to iHPC differentiation in the TLR7.1 model. During *P. yoelii* 17XNL infection, both MyD88 and UNC93B1-dependent endosomal TLRs promote iHPC differentiation and MyD88 participates in the anemia seen in this infection. In vitro, TLR4, TLR7, and TLR9 agonists and IL-1 β can all induce the iHPC- and RPM-expressed transcription factor *Spic* in bone marrow Ly6C^{hi} monocytes, suggesting that MyD88 signaling is sufficient for Spi-C expression. This is a distinct pathway leading to Spi-C induction, as previously, myeloid Spi-C expression was only linked developmentally to RPMs and a subset of bone marrow macrophages, or to heme-induced signals in monocytes (13, 20, 38). Although Spi-C is critical for RPM differentiation and can repress inflammation in intestinal Cx₃CR1⁺ macrophages (38), we found no role for Spi-C in iHPC differentiation or in TLR7-mediated MAS-like disease, though it remains possible that Spi-C subtly affects iHPC function in a manner not yet determined.

Distinct from Spi-C expression, only TLR7 and TLR9 signaling induced Ly6C^{hi} monocyte *Pecam1* transcripts encoding CD31, which is a robust marker for iHPCs in all in vivo settings we examined. Whether TLR3—an endosomal TLR that signals via TRIF—can induce the iHPC program was not tested in vivo. However, Ly6C^{hi} monocytes did not induce *Spic* or *Pecam1* after treatment with poly(I:C) in vitro, suggesting TLR3/TRIF may be unable to signal for iHPC differentiation. Alternatively, the low expression of TLR3 on Ly6C^{hi} monocytes may be insufficient. This leaves us to ask why only TLR7 and TLR9 signaling can cause iHPC differentiation. One possibility is that IRF5, which we show contributes to iHPC differentiation, couples more strongly to these endosomal TLRs than to cell-surface TLRs. Though IRF5 is activated downstream of TLR4, perhaps the balance of NF- κ B and IRF5 activation differs downstream of surface-expressed TLR4 and endosome-expressed TLR7 and TLR9 leading to differential gene transcription. Alternatively, Ly6C^{hi} monocytes may not use IRF5 for TLR4 signaling unlike bone marrow-derived macrophages and other cell types, in which most of the work on IRF5 in LPS responses has been performed (23, 39). Notably, polymorphisms in *IRF5* are linked to increased susceptibility to SLE, a disease associated with innate responses to nucleic acids, and in which IRF5 is constitutively activated in human CD14⁺ classical monocytes (40–44), the corresponding

population to mouse Ly6C^{hi} monocytes. Further studies are required to determine the mechanisms by which IRF5 mediates transcriptional responses for iHPC differentiation. Particularly intriguing is the potential interplay between IRF5, Spi-C, and NF- κ B p65 (38). Additionally, specific signaling components downstream of TLR7 and TLR9 in Ly6C^{hi} monocytes may be involved in iHPC differentiation.

iHPCs are required to cause anemia and thrombocytopenia in TLR7.1 mice, which have a mild lupus-like disease, which we propose here is a model of macrophage activation syndrome (MAS), a complication of SLE and more frequently sJIA. Although sJIA has an autoinflammatory component and can be successfully treated with IL-1-pathway blockers, sJIA patients can develop MAS on these therapies, where MAS can be associated with infection (45). MAS, also called secondary hemophagocytic lymphohistiocytosis (HLH), can also be caused by viral infection. Interestingly, *IRF5* polymorphisms have also been associated with increased risk of these hemophagocytic diseases (25, 26), similar to SLE. Primary or familial HLH, caused by defects in cell-mediated cytotoxicity including mutations in *PRF1* encoding perforin, may be caused by lack of viral clearance by NK cells and CD8⁺ T cells resulting in chronic viral infection (46). Thus, a common thread among MAS or HLH may be chronic or excessive TLR7 and/or TLR9 activation leading to iHPC differentiation.

iHPCs correlate with anemia in a malaria model, suggesting a similarity in mechanisms between MAS and severe malarial anemia. When numbers of *Plasmodium*-induced iHPCs are very low due to MyD88-deficiency, not only was anemia reduced, but also parasitemia, suggesting that iHPCs may be exploited by *Plasmodium* to promote infection. Additionally, “anemia of inflammation”, in which individuals with infections develop mild to moderate anemia, may be a mild form of MAS caused by sustained TLR7 or TLR9 signaling during infection (47). This TLR-driven anemia may have protective roles against secondary bacterial infections after viral infection by reducing the amount of iron available from RBC hemolysis. Our studies not only define a unique monocyte/macrophage induced by TLR7 and TLR9 signaling, but also suggest unexplored avenues to treat MAS, severe malarial anemia, and “anemia of inflammation” by interfering with iHPC generation and function or by their selective depletion.

Materials and Methods

Mice, BM Chimeras, and In vivo Treatments

TLR7.1 mice were obtained from Dr. Sylvia Bolland (NIH) (16), *Spic*^{-/-} (*Spic*^{tm1Kmm}) *Myd88*^{-/-} (*Myd88*^{tm1.1Defr/J}), and *Spic*^{GFP/+} (*Spic*^{tm2.1Kmm}) mice from Jackson Labs (13, 20, 48), *Ccr2*-DTR mice from Dr. Tobias Hohl (MSKCC) (49), and BALB/c, C57BL/6, and B6.SJL mice from Jackson Labs. *Irf5*^{-/-} mice were used on both C57BL/6 and BALB/c backgrounds (23). Bones from *Tlr7*^{-/-}, *MyD88/Trif*^{-/-}, and *Unc93b1*^{-/-} mice were obtained from Dr. Greg Barton (UC Berkeley). All experiments were performed under approved protocols from the Benaroya Research Institute or Feinstein Institute Institutional Animal Care and Use Committee.

Mixed bone marrow chimeras were generated by lethally irradiating (1,000 rad) recipient C57BL/6×B6.SJL F1 mice and reconstituting with a 1:1 ratio of 5×10^6 B6.SJL (CD45.1⁺) and 5×10^6 of either C57BL/6, *Tlr7*^{-/-}, TLR7.1, or *MyD88/Trif*^{-/-} (CD45.2⁺) bone marrow cells. For *Unc93b1*^{-/-}: WT mixed bone marrow chimeras and controls, B6.SJL (CD45.1⁺) recipient mice were lethally irradiated (1,000 rad) and reconstituted with a 1:1 ratio of 5×10^6 C57BL/6×B6.SJL F1 (CD45.1⁺CD45.2⁺) and 5×10^6 *Unc93b1*^{-/-} or C57BL/6 (CD45.2) bone marrow cells. For experiments with TLR7.1/*Ccr2*-DTR mice, mice were injected intraperitoneally with 10 ng DT/g of body weight (List Biological Laboratories) in phosphate-buffered saline every other day for 17 days. In experiments with *Tlr7*^{-/-} and B6.SJL mixed bone marrow chimeras, mice were injected intraperitoneally daily with 100 µg of R848 (Enzo Life Sciences) for 13 days. In experiments with *Irf5*^{-/-}, mice were injected daily for 2 days with 100 µg of R848 (Invivogen). In CpG injection experiments with *Ccr2*-DTR mice, mice were injected intraperitoneally with 40 µg daily of CpG-B (ODN1826) for 5 days (Integrated DNA Technologies). To assess anemia, mice were bled retro-orbitally with heparinized capillary tubes. Blood was run on a Hemavet Hematology Analyzer (Drew Scientific).

In malaria infection experiments, *P. yoelii* 17XNL expressing RFP, provided by S. Kappe and A. Vaughn (50), was passaged through a donor mouse by infection i.p. with 2×10^6 frozen infected RBCs. From day 2 of infection, the donor mouse was monitored for parasitemia by tail vein prick and flow cytometric analysis of blood. When parasitemia reached ~1%, the donor mouse was sacrificed and blood harvested. Recipient mice were each injected i.p. with 1×10^6 infected RBCs.

MCMV (Smith strain) salivary gland viral stocks were provided by Dr. Joe Sun (MSKCC) (51). Five-week-old BALB/c mice were infected with 8×10^3 plaque-forming units (PFU) MCMV i.p. and monitored daily for weight loss. On day 5, mice were sacrificed. Splenocytes were then stained for iHPCs and analyzed by flow cytometry.

Cell isolation, Flow Cytometry, and Cell Sorting

CMPs were isolated as described (9). RPMs and iHPCs were isolated from spleen by digestion in a cocktail of Liberase TL (Sigma) at 0.17 mg/ml and DNase I (Sigma) at 40 µg/ml in complete RPMI (Hyclone). Bone marrow cells were isolated by centrifugation. Splenocytes and bone marrow cells were blocked with polyclonal rat IgG (65 µg/ml, Sigma) and purified anti-mouse Ter-119 (Ter-119, 71.4 µg/ml, Biolegend) prior to cell surface staining with fluorescently labeled antibodies as indicated in Supplementary Materials and Methods. Cells were then washed and incubated in Fixable Viability Dye eF780 (eBioscience) or Live/Dead Fixable Blue Dead Cell Stain (Invitrogen). Cells were washed and prepared for intracellular staining with Fixation and Permeabilization buffer (BD Biosciences), washed in Perm/Wash buffer (BD Biosciences) and then stained with fluorescently labeled anti-Ter-119 to detect cells that had phagocytosed RBCs. To accurately assess phagocytosis, some samples were blocked intracellularly with purified anti-mouse Ter-119 (25 µg/ml) prior to intracellular staining with fluorescently labeled anti-Ter-119. To assess spleen and bone marrow cells without Ter-119 stain, cells were blocked with polyclonal rat IgG and polyclonal mouse IgG (65 µg/ml, Sigma), stained with appropriate

antibodies, and then fixed with 2% paraformaldehyde. Data were acquired on an LSRII or FACSCanto (BD Biosciences) and analyzed using FlowJo (Tree Star).

For isolation of iHPCs, RPMs, and monocytes for quantitative real-time PCR and microscopic analysis, splenocytes isolated as above were stained with APC-conjugated rat anti-mouse SIRP α (P84, 1 μ g/ml), incubated with anti-APC beads (Miltenyi), and positively selected on LS columns (Miltenyi). Following isolation, cells were then stained as indicated in Supplemental Materials and Methods and sorted on a FACS Aria (BD Biosciences). For experiments requiring only monocytes, bone marrow was depleted using biotinylated antibodies to hamster anti-mouse CD3e (eBio500A2, eBioscience), rat anti-mouse CD19 (6D5, Biolegend), rat anti-mouse Ly6G (1A8, Biolegend), and mouse anti-mouse NK1.1 (PK136, eBioscience) (all at 5 μ g/ml), anti-biotin beads (Miltenyi), and LS columns (Miltenyi). Negatively selected bone marrow was then stained with rat anti-mouse Ly6C (HK1.4), rat anti-mouse Siglec-F (E50–2440), rat anti-mouse Ly6G (1A8), rat anti-mouse/human CD11b (M1/70), rat anti-mouse MHCII (M5/114.15.2), and hamster anti-mouse CD11c (N418). Cells were then sorted on a FACS Aria (BD Biosciences) as in Fig. S3.

In Vitro Cell Culture

Sorted bone marrow CMPs were plated as previously described (9). CMPs (2,500–20,000) were plated per well in 96-well plates in complete serum-free StemPro-34 media (Life Technologies) with 20 ng/ml of stem cell factor (Peprotech) for all experiments. Unless otherwise noted, 1 μ g/ml of R848 (Invivogen) and 20 ng/ml of M-CSF (Peprotech) were used. For flow cytometric quantification of CD11b⁺ F4/80⁺ cells, adherent cells were isolated using cell dissociation buffer (Life Technologies) and stained with antibodies to CD11b and F4/80. Cell yield was quantified using polystyrene microspheres (Polysciences) and flow cytometry as the number of CD11b⁺F4/80⁺ events per well divided by the number of polystyrene bead events per well multiplied by the total number of polystyrene beads per well.

For gene expression analysis, sorted monocytes were plated in either media alone, 100 ng/ml of LPS (List Biologicals), 3 μ M CpG-C ODN2395 (Integrated DNA Technologies), 10 μ g/ml of R848 (Invivogen), 40 ng/ml of MCSF (Peprotech), 100 ng/ml of IFN γ (Peprotech), 100 ng/ml of IL-1 β (Miltenyi), or 25–50 μ g/ml of Poly(I:C) (Invivogen).

Hemophagocytosis Assay

CMPs were sorted as above and then placed in culture at 10,000 cells/well in either 10 μ g/ml of R848 or 40 ng/ml of MCSF. On day 4, cells were harvested from wells with cell dissociation buffer (Gibco), washed in Dulbecco's Modified Eagle Medium (Gibco) supplemented with 10% fetal bovine serum (Sigma-Aldrich), plated at 50,000 cells per well in non-tissue culture treated flat bottom 96-well plates, and rested overnight. On day 5, heparinized blood was harvested from a C57BL/6 mouse, and 40 μ l of whole blood (or approximately 4×10^8 RBCs) were then washed in PBS and resuspended at 2×10^7 /ml in 20 ml of PBS containing 5 μ M CFSE (Sigma-Aldrich) and incubated at 37°C for 10 minutes while shaking to obtain CFSE-labeled RBCs. Following incubation, the reaction was stopped with DMEM with 10% FBS for 10 minutes on ice, and then washed three times.

R848-differentiated macrophages were pretreated with 2 μ M cytochalasin D (Calbiochem) or DMSO (Fisher) vehicle for 30 minutes and 10 μ g/ml of purified anti-SIRP α antibody (P84) for 15 minutes, prior to the addition of CFSE-labeled RBCs. Following a 15-minute incubation with RBCs, assay wells were washed with cold PBS and extracellular RBCs were lysed with cold ACK lysis buffer (Lonza) for 5 minutes on ice. Cells were then washed with cold DMEM with 10% FBS, followed by cold PBS. Macrophages were then harvested from assay plates with cell dissociation buffer (Gibco, ThermoFisher) at 37°C on a shaker for 10 min. Cells were blocked for Fc receptor binding with polyclonal rat IgG and polyclonal mouse IgG (65 μ g/ml, Sigma) and stained for CD11b and F4/80 for 20 minutes. Samples were then washed, fixed with 2% paraformaldehyde, and analyzed on a FACSCanto (BD Biosciences) and using FlowJo software (Tree Star).

Microscopy

RPMs and iHPCs were sorted from spleens of TLR7.1 and WT mice, cytospun onto slides (Cytospin 4, Thermo Scientific), and stained by hematoxylin and eosin. At least two fields per sample were quantified for phagocytic index using a Leica DM2500 microscope with SPOT Software 5.1 and SPOT Insight Wide-field 4 Mp Monochrome FireWire Digital Camera. A total of 43–409 cells per sample were counted. The phagocytic index was calculated by using the following formula: $PI = (\% \text{ phagocytic cells containing } 1 \text{ RBC}) \times (\text{mean number of RBC/phagocytic cell containing RBCs})$.

Quantitative RT-PCR

RNA was generated using RNeasy Plus Mini Kit and RNeasy MinElute Cleanup kit (Qiagen). cDNA was synthesized using Primescript (Takara) and Invitrogen reagents with random hexamers and OligoDT primers. qPCR was performed using SYBR green reagents (Takara) on a 7500 Fast Real-Time PCR System (Applied Biosystems). Arbitrary units were calculated using the CT method normalized to HPRT. The values were then normalized again by setting either media alone (--) or monocyte values to 1.

RNA-Seq and Bioinformatic Analysis

CMP+R848 vs. CMP+M-CSF RNA-Seq: Samples were generated by sorting CMPs and plating 10,000 cells per well of a 96-well plate in complete serum-free StemPro 34 (Gibco) media with 20 ng/ml Stem Cell Factor (Peprotech) for 4 days with 1 μ g/ml of R848 (Invivogen) or 20 ng/ml of MCSF (Peprotech). At day 4, cells were washed with Dulbecco's Modified Eagle Medium (Gibco) supplemented with 10% fetal bovine serum (Sigma-Aldrich), rested for 24 hours and isolated using cell dissociation buffer. Samples were sorted as propidium iodide⁻ CD11b⁺F4/80⁺ cells directly into RLT plus lysis buffer (Qiagen). RNA was generated using RNeasy kit and MinElute cleanup kit (Qiagen). RNA from three independent sorts for each MCSF and R848-differentiated macrophages were used for RNA sequencing.

RNA-Seq libraries were constructed from 100 ng of RNA using the TruSeq RNA Sample Preparation V2 kit (Illumina). Libraries were clustered on a flowcell using the TruSeq SR Cluster Kit, v3 using a cBot (Illumina), followed by single read sequencing on a HiSeq2500

(Illumina) for 100 cycles. FASTQ files were downloaded from <https://basespace.illumina.com>.

Libraries were processed via Galaxy on a local computer cluster. Libraries were aligned via TopHat (v1.4.1) to Ensembl's Mus musculus GRCm38.78 gtf (http://may2012.archive.ensembl.org/Mus_musculus/Info/Index). The `--single-paired` flag was set to "single," whereas all other TopHat parameters were set to defaults. HTSeq-count (52) was used to generate gene counts with mode as "Intersection (nonempty)" and Minimum alignment quality set to 0; all others were set to default parameters.

Analysis of the 37,991 Ensembl ID count data was performed using the edgeR package (53) in the R software environment (R Core Team (2017). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. <https://www.R-project.org/>). For each gene, a negative binomial general linear model (54) appropriate for count data was used for the two-group comparisons of R848 versus MCSF. The Ensembl IDs were filtered to those that had a TMM normalized count of at least one in at least one library, which resulted in 13,690 Ensembl IDs used in the general linear model. The two-group comparison had an 18% biological coefficient of variation and 1,833 protein coding Ensembl IDs had a false discovery rate less than 0.05 and fold change greater than 2 (in either direction) (Shilin Zhao, Yan Guo, Quanhu Sheng and Yu Shyr (2015). heatmap3: An Improved Heatmap Package. R package version 1.1.1. <https://CRAN.R-project.org/package=heatmap3>).

Tissue macrophage gene signatures were identified in Gautier et al. 2012 (14). Analysis of tissue macrophage signature overlap was conducted by comparing the number of genes significantly upregulated (>2-fold) in R848-derived macrophages versus MCSF-derived macrophages to the number of significantly enriched genes in each tissue macrophage signature(14) using an online tool (http://nemates.org/MA/progs/overlap_stats.html).

iHPC, RPM and Ly6C^{hi} monocyte RNA-Seq: Five hundred cells were sorted into lysis buffer from the SMART-Seq v4 Ultra Low Input RNA Kit for Sequencing (Takara). Reverse transcription was performed followed by PCR amplification to generate full-length amplified cDNA. Sequencing libraries were constructed using the NexteraXT DNA sample preparation kit (Illumina) to generate Illumina-compatible barcoded libraries. Libraries were pooled and quantified using a Qubit® Fluorometer (Life Technologies). Dual-index, single-read sequencing of pooled libraries was carried out on a HiSeq2500 sequencer (Illumina) with 58-base reads, using HiSeq v4 Cluster and SBS kits (Illumina) with a target depth of 5×10^6 reads per sample. Basecalls were processed to FASTQs on BaseSpace (Illumina), and a base call quality trimming step was applied to remove low-confidence base calls from the ends of reads.

Libraries were processed via Galaxy as above. Analysis of the 37,991 Ensembl ID count data was performed using voom from the limma package updated for RNA-sequencing experiments (55) in the R software environment. The Ensembl IDs were filtered to those that encoded for protein coding genes, and had a TMM normalized count of at least one count per million in at least 10% of the libraries, which resulted in 12,856 Ensembl IDs used in the

general linear model. Samples were removed from analysis if the number of aligned reads was less than 500,000. Two-group comparisons were filtered for a false discovery rate of 0.05 after multiple testing correction, and a fold change of 1.5 or greater. Heatmaps were constructed using the heatmap3 package (54).

RNA-Seq data are available in GEO SuperSeries GSE117718 containing CMP dataset (GSE70520) and iHPC dataset (GSE117711).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. TLR7 promotes RPM-like hemophagocyte development in vitro.

(A) Representative flow cytometric staining of CMPs cultured with SCF or SCF+R848. (B-D) RNA-Seq analysis of CD11b⁺F4/80⁺ macrophages sorted from CMPs differentiated in R848 or MCSF. (B) Of the 22,707 total protein coding genes, 813 were upregulated and 1,020 were downregulated in R848-differentiated macrophages compared to MCSF-differentiated macrophages (2-fold, FDR 0.05). Three independent biological replicates were sequenced for each condition. (C) Red bars indicate the percent of genes in a tissue macrophage core transcriptional signature (14) that were significantly increased in R848-differentiated compared to MCSF-differentiated macrophages. Black line indicates -log p-value calculated using exact hypergeometric probability with a normal approximation. (D) Heat map of increased genes (2-fold, FDR 0.05) in R848-differentiated compared to MCSF-differentiated macrophages in RPM core signature. (E) R848-differentiated macrophages were treated with or without cytochalasin D (CytoD) then allowed to phagocytose CFSE-labeled RBC for 15 min at indicated ratios. Percent of CD11b⁺F4/80⁺ macrophages that had phagocytosed RBC is shown. Data are representative of four experiments, n=3 technical replicates per condition/experiment. Mean values+SD (E) are shown.

Figure 2. TLR7 signaling drives formation of hemophagocytes distinct from RPMs in vivo. (A-D) Splenocytes from WT and TLR7.1 mice were surface-stained for CD11b, CD45.2, F4/80, Ly6G and Siglec-F, and then intracellularly stained with mAb to Ter-119 to detect leukocytes that had phagocytosed RBCs. (A) Representative flow cytometry pre-gated as live singlets, CD45.2⁺ cells. Prior to intracellular staining with fluorescently labeled anti-Ter-119, some samples were blocked with unconjugated Ter-119 (control). Other samples were not blocked prior to staining (Ter-119). (B) Frequency and number of CD45.2⁺Ly6G⁻Siglec-F⁻Ter-119⁺ cells, and mean fluorescence intensity (MFI) of Ter-119 staining in CD45.2⁺Ter-119⁺ cells were then quantified. Data are representative of four experiments. (C) CD11b and F4/80 expression on CD45.2⁺Ly6G⁻Siglec-F⁻Ter-119⁺ splenocytes. iHPCs are in blue and RPMs are in red. Data are representative of four experiments. (D) RPMs were gated as CD45.2⁺Ly6G⁻Siglec-F⁻Ter-119⁺ cells that were CD11b^{lo/int}F4/80^{hi} and iHPCs were gated as CD45.2⁺Ter-119⁺ cells that were CD11b^{int/hi}F4/80^{lo}. Histograms of indicated surface staining are shown. Data are representative of four experiments. (E) Percentage of RPMs or iHPCs that were Ter-119⁺ from WT and TLR7.1 mice quantified by flow cytometry. Data are representative of four experiments, n=5 per group. (F and G) RPMs and iHPCs were FAC-sorted and stained by H&E. Intracellular RBCs were quantitated by

microscopy. (G) Phagocytic index (number of intracellular RBCs per 100 cells) (left) and percentage of cells with at least one RBC (right) were calculated. Data are representative of two experiments, n=3–4 per group. (B) mean±SEM, (F and G) mean+SEM, (B) each symbol represents an individual mouse. *p<0.05, ** p<0.01, ***p<0.001, ****p<0.0001, two-tailed, unpaired Student's *t*-test (B). one-way ANOVA with Tukey's post test (E).

Figure 3. iHPCs differentiate in response to cell-intrinsic TLR7 signals.

A) The ratio of TLR7.1 to WT BM-derived cells in mixed bone marrow chimeras of indicated populations after reconstitution. Data are representative of two experiments with $n=8-10$ per experiment. B) The ratio of WT to *Tlr7*^{-/-} BM-derived cells in mixed bone marrow chimeras injected with the TLR7 agonist R848 (right) or PBS (left) for 13 days. Data are representative of two experiments with $n=3-5$ per group per experiment. (A, B) mean $\pm$ SEM, (A, B) each symbol represents an individual mouse. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$, one-way ANOVA with Dunnett's post test (A and B).

Figure 4. iHPCs are derived from Ly6C^{hi} monocytes.

(A) Splenic monocytes (live singlets, CD11b⁺F4/80⁻Ly6G⁻Ly6C^{hi} or CCR2⁺ cells) (black) and iHPCs (live singlets, F4/80^{lo}Ly6G⁻Ter-119⁺VCAM1^{lo} or CD31^{hi} cells) (blue) were assessed for the expression of the cell surface proteins indicated (solid lines) compared to fluorescence minus one (FMO) control stains (dashed lines). Data are representative of three experiments. (B) Bone marrow Ly6C^{hi} monocytes were sorted from WT B6 mice and cultured for 21 hours with media alone (--) or with R848. *Spic*, *Pecam1*, *Ccr2*, and *Ly6c1* transcripts were quantified by qPCR. Data are representative from five experiments with n=3 per experiment. (C, D) Ccr2-DTR⁻, WT/Ccr2-DTR⁺, TLR7.1/Ccr2-DTR⁻, and TLR7.1/Ccr2-DTR⁺ mice (n=5–7 mice per group) were injected with DT every other day for 17 days. (C) Representative flow cytometry of Ter-119⁺ hemophagocytes pre-gated on live singlets, CD45.2⁺Ly6G⁻Siglec-F⁻ cells from the spleens of TLR7.1/Ccr2-DTR⁻ and TLR7.1/Ccr2-DTR⁺ mice determined by flow cytometry. (D) Frequency and number of the indicated cell populations were quantitated from the spleens of Ccr2-DTR⁻, Ccr2-DTR⁺,

TLR7.1/Ccr2-DTR⁻, and TLR7.1/Ccr2-DTR⁺ mice by flow cytometry. Data are combined from three experiments. (E) Bone marrow Ly6C^{hi} monocytes were sorted from WT B6 mice and cultured for 21 hours with media alone (--), R848, LPS, or CpG. *Spic*, *Pecam1*, *Ccr2*, and *Ly6c1* transcripts were quantified by qPCR. Data are representative from two experiments. (F and G) RNA-Seq analysis of RPMs and Ly6C^{hi} monocytes sorted from spleens of WT B6 mice and TLR7.1 mice and iHPCs from spleens of TLR7.1 mice. n=5 (TLR7.1 iHPC), n=4 (WT and TLR7.1 RPM), n=6 (WT and TLR7.1 Mono). (F) PCA of indicated populations. (G) Heat map of DEG between the three populations (RPMs, Ly6C^{hi} monocytes, and iHPCs) sorted from TLR7.1 mice. Mean values+SEM (D), ± SEM (B, E). *p<0.05, ** p<0.01, ***p<0.001, ****p<0.0001, two-tailed, unpaired Student's *t*-test (B) and Mann–Whitney *U* test (D).

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Figure 5. Monocyte-derived iHPCs drive anemia.

(A) RBC count, hemoglobin levels, and hematocrit from 3-month-old WT and TLR7.1 mice.

Each symbol represents an individual mouse, n=8–12 mice per group. (B) Correlation between RBC count and number (left) and frequency (right) of splenic Ter-119⁺ iHPCs in TLR7.1 mice. (C) RBC count of TLR7.1 WT, TLR7.1 Spic^{+/-}, TLR7.1 Spic^{-/-}, and control mice that were bled prior to 8 weeks and between 9 and 13 weeks of age. n=7–14 mice per group. (D) TLR7.1/Ccr2-DTR^{-/-} and TLR7.1/Ccr2-DTR^{+/+} mice were treated with DT every other day for 17 days beginning when RBC count was below 8. RBC count was measured at indicated times. n=5–7 mice per group. (E) Ccr2-DTR^{-/-} and Ccr2-DTR^{+/+} mice were treated with DT every day for 6 days and CpG daily starting one day after beginning DT treatment. RBC count was measured prior to and at the end of treatment. Data are representative of two experiments, n=3 (No Tx) and n=7 (CpG) mice per group. (F) Platelet counts in TLR7.1/Ccr2-DTR^{-/-} and TLR7.1/Ccr2-DTR^{+/+} mice treated as in (D). Mean values ± SEM (A, C-F) are shown. (A and B) each symbol represents an individual mouse. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, ns not significant Mann-Whitney (A), Wilcoxon paired t-test of Days -1 and +17 for TLR7.1 experiment and -1 and +5 for CpG injection (D-F).

Figure 6. iHPC differentiation depends on IRF5.

(A) Bone marrow Ly6C^{hi} monocytes were sorted from WT and *Irif5*^{-/-} mice and cultured for 21 hours with media alone (--) or with R848. *Spic*, *Pecam1*, *Ccr2*, and *Ly6c1* transcripts were quantified by qPCR. Data are representative from two experiments with n=2 to 4 per group per experiment. (B and C) WT and *Irif5*^{-/-} mice were injected with R848 i.p. daily for 2 days. Splenocytes were analyzed by flow cytometry. (B) Representative flow plots of WT and *Irif5*^{-/-} CD11b⁺CD31⁺ iHPCs (gated on live singlets, CD45.2⁺F4/80⁻Ly6G⁻Siglec-F⁻ cells). (C) Frequency (left) and number (right) of iHPCs in WT and *Irif5*^{-/-} spleens. Data are representative from two experiments with n=4 per group per experiment. Mean values±SEM (A and C) are shown. (A and C) each symbol represents an individual mouse. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, two-tailed, unpaired Student's *t*-test (A and C).

Figure 7. iHPC development during malaria infection is dependent on Myd88 and endosomal TLRs.

(A-E) B6 mice were injected with 1×10^6 *P. yoelii* 17XNL-infected RBCs (closed squares) or PBS (open circles) and analyzed at indicated days (A, B, D) or day 12 of infection (C). (A) Parasitemia as measured by flow cytometry of RFP-expressing *P. yoelii* 17XNL, and RBC and platelet count during the course of infection. (B) Percent (left) and number (right) of intracellular Ter-119⁺ cells of total CD45⁺ splenocytes. (C) Gated CD31⁺CD45.2⁺Ly6G⁻Siglec-F⁻Ter-119⁺ iHPCs (blue gate) and RPMs (red gate) on day 12 of infection. (D) iHPC frequency (left) and number (right) during the course of infection. (E) Correlation of RBC count and iHPC number on all days. Data are representative of two experiments, $n=3$ (PBS), 5–6 (*P. yoelii* 17XNL) mice per group. (F and G) WT B6 and *Myd88*^{-/-} mice were infected with 1×10^6 *P. yoelii* 17XNL-infected RBCs and analyzed at day 12 of infection (F) or the indicated days (G). (F) Gated CD11b⁺CD31⁺ iHPCs (Gated on live singlets, CD45.2⁺F4/80⁻Ly6G⁻Siglec-F⁻) on day 12 of infection. (G) iHPC frequency and number per spleen at day 12 of infection (left); RBC count and parasitemia at the indicated days (right). Data

are representative of two experiments, $n=5$ (WT), and $n=4-5$ (*Myd88*^{-/-}) mice per group. (H, I) The ratio of WT to *Unc93b1*^{-/-} (H) or WT to *Myd88*^{-/-} *Trif*^{-/-} (I) BM-derived cells in mixed bone marrow chimeras of indicated populations before and on day 8 after infection with 1×10^6 *P. yoelii* 17XNL-infected RBCs. Ly6C^{hi} monocytes pre-infection are from blood (open circles). Ly6C^{hi} monocytes post-infection (black circles) and iHPCs post-infection (blue circles) are from spleen. Data are representative of two experiments. Mean values $\pm$ SEM (A, B, D, G, H, and I) are shown. (E, G left, H, and I) Each symbol represents an individual mouse. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$, two-tailed, unpaired Student's *t*-test (A, B, and G), Linear regression (E), one-way ANOVA with Tukey's post test (H, I).