PPARγ is essential for the development of bone marrow erythroblastic island macrophages and splenic red pulp macrophages

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Tissue-resident macrophages play a crucial role in maintaining homeostasis. Macrophage progenitors migrate to tissues perinatally, where environmental cues shape their identity and unique functions. Here, we show that the absence of PPARγ affects neonatal development and VCAM-1 expression of splenic iron-recycling red pulp macrophages (RPMs) and bone marrow erythroblastic island macrophages (EIMs). Transcriptome analysis of the few remaining Pparg-deficient RPM-like and EIM-like cells suggests that PPARγ is required for RPM and EIM identity, cell cycling, migration, and localization, but not function in mature RPMs. Notably, Spi-C, another transcription factor implicated in RPM development, was not essential for neonatal expansion of RPMs, even though the transcriptome of Spic-deficient RPMs was strongly affected and indicated a loss of identity. Similarities shared by Pparg- and Spic-deficient RPM-like cells allowed us to identify pathways that rely on both factors. PPARγ and Spi-C collaborate in inducing transcriptional changes, including VCAM-1 and integrin α6 expression, which could be required for progenitor retention in the tissue, allowing access to niche-related signals that finalize differentiation.

Introduction
Tissue-resident macrophages are immune phagocytes present in almost all tissues (Hume and Gordon, 1983; Hume et al., 1983; Lee et al., 1985). In addition to general functions, like pathogen clearance and tissue homeostasis (for a review, refer to Gordon, 2007, and Murray and Wynn, 2011), tissue-resident macrophages are specialized to perform additional environment-imposed functions (Chow et al., 2011; Parkhurst et al., 2013). The mechanisms underlying this adaptation to the tissue’s needs are poorly understood. Tissue macrophages develop from fetal progenitors and are able to homeostatically self-renew in adulthood (Ginhoux et al., 2010; Hashimoto et al., 2013; Jakubzick et al., 2013; Schulz et al., 2012; Yona et al., 2013). Upon tissue seeding, which occurs before birth, progenitor cells receive environment-specific signals, such as cytokines or metabolites, which induce signaling cascades and a unique differentiation program, leading to the development of specialized macrophages (Amit et al., 2016; Gautier et al., 2012b; Glass and Natoli, 2016; Gosselin et al., 2014; Lavin et al., 2014; Lavin et al., 2015). PU.1—encoded by the Spi1 gene and a member of the ETS domain-containing family—has been shown to be an essential transcription factor for macrophage development (Schulz et al., 2012). It binds to both common and subset-specific genomic locations and is required for collaborative interactions with alternative sets of transcription factors induced by environmental triggers in each tissue-specific macrophage subset (Gosselin et al., 2014).

While fate mapping studies have helped to understand the origin of macrophages, transcriptome and enhancer region analyses have provided crucial information about tissue-specific gene signatures (Gautier et al., 2012b; Gomez Perdiguero et al., 2015; Gosselin et al., 2014; Hoefel et al., 2015; Lavin et al., 2014). For example, TGFβ-induced SMAD2/3 activation is crucial for microglia development (Abutbul et al., 2012; Butovsky et al., 2014), while peritoneal macrophages depend on GATA6, which is induced by retinoic acid binding to the retinoic acid receptor-β (Okabe and Medzhitov, 2014). Terminal differentiation of osteoclasts, a macrophage subset residing in bones and responsible for bone resorption, depends on NFATc1 activation via receptor activator of NF-κB ligand signaling (Takayanagi et al., 2002). In lungs, GM-CSF production by lung epithelial cells induces peroxisome proliferator-activated receptor-γ (PPARγ), which is indispensable for alveolar macrophage (AM) differentiation.
from fetal monocytes (Guilliams et al., 2013; Schneider et al., 2014). The spleen contains several macrophage subsets, including marginal zone macrophages, marginal metallophilic macrophages (MMMs), and red pulp macrophages (RPMs). Marginal zone macrophages and MMMs have been shown to be dependent on nuclear liver X receptor-α, which is activated by a so-far-unknown trigger (A-Gonzalez et al., 2013). RPMs develop in an M-CSF-dependent manner and rely on Spi-C, a PU.1-related factor (Kohyama et al., 2009; Kurotaki et al., 2011). Similarly, Spi-C is needed for a population of vascular cell adhesion molecule-1 (VCAM-1)-positive bone marrow macrophages (Haldar et al., 2014), also known as bone marrow erythroidlastic island macrophages (EIMs) due to their function in facilitating iron recycling from senescent RBCs, similar to RPMs in the spleen (Kurotaki et al., 2015). Both RPMs and EIMs constitutively degrade hemoglobin and metabolize the highly oxidative intermediate product of this degradation, heme. The BTB domain and CNC homologue 1 (Bach1) transcription regulator represses Spi-C expression in monocytes. It has been shown that heme can bind to Bach1, and heme-bound Bach1 is marked for proteasome degradation (Haldar et al., 2014).

In this study, we describe a previously unknown crucial role of PPARγ in RPM and EIM development. Although this factor is the main driver of perinatal AM differentiation, the programs induced by PPARγ in lungs and spleen are distinct and, in the latter case, are not related to lipid metabolism. Moreover, we show that upon tissue seeding, developing RPMs downregulate migration-related markers and strongly upregulate integrin αv, which is uniquely expressed by this macrophage subset and is correlated with cell retention (Aziz et al., 2017). These results are an example of how niche-specific signals collaborate with the transcriptional regulator PPARγ to promote alternative differentiation programs.

Results
Spi-C is not essential for neonatal RPM development
Spi-C is a signature transcription factor of RPMs, and adult Spi-C-deficient mice have been reported to lack RPMs and EIMs (Haldar et al., 2014). Since most resident macrophages develop perinatally from fetal precursors, we have revisited the role of Spi-C for RPMs and compared newborn Spi-C−/− with WT counterparts. First, we confirmed a strong reduction of RPMs and EIMs in adult Spi-C−/− mice (Figs. 1 A and S1 B). Strikingly, the number of RPMs and EIMs in 7-d-old mice were unaffected by the lack of Spi-C (Figs. 1 B and S1 C). At the age of 2 and 4 wk, RPM numbers were already significantly reduced (Fig. S1 D), although not as striking as in adults, indicating a loss of these populations after their neonatal expansion. The transcriptome analysis of RPMs from 7-d-old pups showed that the expression of 1,447 genes in Spi-C-deficient RPMs was changed more than 2.8-fold (false discovery rate [FDR] < 0.05). The top 50 differentially expressed genes (DEGs) are listed in Fig. 1 C. Gene ontology (GO) analysis further identified the altered biological pathways in Spi-C-deficient cells, including decreased processes of oxidation reduction, cellular oxidant reduction or heme catabolism and increased cell adhesion, signal transduction, or response to stimulus (Fig. 1 D). Moreover, comparison of DEGs (log2 ratio > 1.5) to signature genes, defined for different tissue-resident macrophage subsets by Lavin et al. (2014), revealed decreased expression of many RPM-related signature genes and increased expression of genes related to macrophages from other tissues, like colon or brain (Fig. 1 E). Previously described signature genes, like Spi-C, VCAM-1, and TREML4 (Haldar et al., 2014), present in the splenic macrophage signature identified by Lavin et al. (2014) were all significantly reduced (Fig. 1 F). Interestingly, we noted a significant decrease in PPARγ mRNA (Fig. 1 F) and protein expression (Fig. 1, G and H; and Fig. S1 E) in Spi-C-deficient RPMs and EIMs, possibly indicating a role for PPARγ in the development of iron-recycling macrophages, similar to its role in AMs (Schneider et al., 2014). These results show that in the absence of Spi-C, RPM- and EIM-like cells can be produced but they could be functionally altered and are not maintained after initial expansion.

Reduced EIMs and iron-recycling RPMs in the absence of PPARγ
To assess whether PPARγ is important for RPM development, we used a Vav1-Cre/Ppargfl/fl mouse line that efficiently deletes PPARγ in all hematopoietic cells, including differentiating fetal monocytes (Schneider et al., 2014). Development of RPMs and EIMs—characterized as F4/80+CD11b+VCAM-1− cells—is considerably impaired in adult Vav1-Cre/Ppargfl/fl mice (Fig. 2, A and B). Notably, Cdilc-Cre/Ppargfl/fl mice and LysM-Cre/Ppargfl/fl mice showed no significant reduction of RPMs and EIMs (Fig. S2, A–C), most probably due to poor Cdilc and LysM promoter-driven Cre recombinase expression and accordingly inefficient PPARγ deletion in macrophages (Schneider et al., 2014). In contrast, Vav1-Cre expression in Rosa26–RFP reporter mice induced deletion of a loxP-flanked stop cassette and RFP expression in >95% of RPMs without Cre toxicity (Fig. S2 D). Immunohistology of spleens confirmed the almost complete absence of F4/80+ cells in the red pulp of Vav1-Cre/Ppargfl/fl mice, while CD169+ (MOMA+) MMMs, localized at the interface of white pulp and red pulp, were unchanged from WT controls (Fig. 2 C). RPMs are critical for phagocytosis of senescent RBCs and iron recycling. Nevertheless, RBC number, mean corpuscular volume, hematocrit (HCT), hemoglobin, and reticulocyte counts were unaffected in the circulating blood of Vav1-Cre/Ppargfl/fl mice, while CD169+ (MOMA+) MMMs, localized at the interface of white pulp and red pulp, were unchanged from WT controls (Fig. 2 C). RPMs require PPARγ intrinsically for neonatal development
Most tissue macrophages arise during perinatal development (Ginhoux and Guilliams, 2016). Indeed, we noted a 20-fold expansion of RPMs between days 2 and 5 (Fig. 3 A), concomitant with the increase in PPARγ expression (Fig. 3 B). To assess whether PPARγ regulates postnatal RPM development, we analyzed RPM populations in Vav1-Cre/Ppargfl/fl mice and Ppargfl/fl littermate controls at different days after birth. While the postnatal expansion of RPMs was strongly reduced in the absence of PPARγ (Figs. 3 A and S3 A), this reduction did not
Figure 1. RPM numbers in Spic−/− mice are not affected early after birth. (A and B) Flow cytometry of spleens from 10-wk-old (A) and 5–7-d-old (B) Spic+/+ and Spic−/− mice; plots of a representative individual (left) and bar graphs (right) showing RPM counts (upper) and their frequencies of CD45+ cells (lower). RPMs were gated as shown in Fig. S1 A. (C) Heat map showing the top 50 DEGs between Spic+/+ and Spic−/− RPMs (according to absolute values of gfold). Cells were isolated from 7-d-old mice. (D) Upregulated (red) and downregulated (blue) GO terms for Biological Process (BP) from comparison of Spic−/− over Spic+/+ RPMs (P < 0.01). (E) Bar graphs showing upregulated (red) and downregulated (blue) genes from a comparison of Spic−/− over Spic+/+ RPMs \( \log_{2} \).
significantly affect the total number of splenocytes (Fig. S3 B). The frequency of Ly6C+ monocytes was increased in neonatal Vav1-Cre/Ppargfl/fl mice and inversely corresponded to the decrease in RPMs (Fig. S3, C and D). Moreover, the few remaining RPM-like cells in Vav1-Cre/Ppargfl/fl neonates entered cell cycling, indicated by Ki67 expression, and proliferated.

![Figure 2](https://doi.org/10.1084/jem.20191314)

**Figure 2. Development of iron-recycling macrophages is abrogated in the absence of PPARγ.** (A and B) Flow cytometry plots of spleen (A) and bone marrow (B) from Ppargfl/fl and Vav1-Cre/Ppargfl/fl mice (7–11 wk old; pooled from three experiments indicated by different colors) and bar graphs showing RPM/EIM counts and their frequencies of CD45+ cells. RPMs/EIMs were pregated as live CD45+ cells. (C) Images of immunohistochemistry staining of B220 (B cell marker), MOMA (metallophilic macrophage marker), and F4/80 (RPM marker) on splenic sections. White scale bars, 25 µm. (D) Comparison of the numbers of RBCs and reticulocytes, hemoglobin (HGB), HCT, and mean corpuscular volume (MCV) of RBCs from blood test of Ppargfl/fl and Vav1-Cre/Ppargfl/fl mice. (E) Representative microscopic images of splenic sections demonstrating ferric iron deposits by Perls’ Prussian Blue stain. White scale bars, 50 µm. (F) Bar graph (left) and rug plot (right) presenting the quantification of blue-stained area from two biological replicates. The presented data are pooled from three independent experiments (A and B; mean and SD of 10 mice per group) or are representative of two independent experiments (D; mean and SD of three to five mice per group). *, P < 0.05; ***, P < 0.001; ****, P < 0.0001 (unpaired two-tailed Student’s t test).
Figure 3. **RPMs require PPARγ intrinsically for their development.** (A) Total RPM counts from *Pparg*fl/fl and Vav1-Cre/*Pparg*fl/fl mice (gated as shown in Fig. 2A) at indicated days after birth (postnatal day [PND]). (B) PPARγ expression by RPMs from representative WT (*Pparg*fl/fl) mice at indicated PNDs and in adults shown as histograms (left) and as averages of MFI (normalized to FMO controls) from groups of WT and KO mice (right). (C and D) Bar graphs showing percentages of Ki67+ (C) and EdU+ (D) RPMs at indicated time points. (E) VCAM-1 expression gated on F4/80+ RPMs shown as dot plots from representative individuals at PND 7 (left) and bar graphs showing MFI (mean and SD) of groups of *Pparg*fl/fl and Vav1-Cre/*Pparg*fl/fl at PND 3, 5, and 7 (different color symbols indicate separate experiments; right). (F) Graphical scheme of experiments aiming at blocking (G) and inducing (H) PPARγ expression. (G) Total RPM counts from adult C57BL/6 mice treated i.p. with 1 mg/kg of PPARγ inhibitor GW9662 (left) or with 10 mg/kg of PPARγ ligand rosiglitazone (right) for 7 consecutive days prior to analysis at days indicated in the scheme. (H) Total RPM counts, frequency of Ki67+, and normalized MFI of PPARγ in RPMs from adult C57BL/6 mice at day 4 after hydrodynamic IL-4 gene delivery (plasmid pTT5-Il4). (I) Total RPM counts from mice of indicated genotypes. All mice were 8–12 wk old; RPMs gated as in Fig. 2A. (J) Graphical presentation of experiments designed to test the fetal monocyte intrinsic requirement of PPARγ for RPM development. CD45+ cells were sorted from fetal livers of E14.5 embryos (CD45.1) and subsequently transferred to *Pparg*fl/fl and Vav1-Cre/*Pparg*fl/fl newborns. Analysis of the RPM reconstitution was performed 8 wk after transfer. (K) Dot plots showing RPM origin in recipient mice 8 wk after transfer. RPMs are gated as live CD45.1 cells.
CD11b<sup>+</sup>F4/80<sup>+</sup>VCAM-1<sup>+</sup>CD11c<sup>−</sup>; red gates indicate CD45.1<sup>+</sup> RPMs (derived from transferred fetal progenitors). (I) RPM counts of mice supplemented with fetal monocyte precursors or not (left), and frequencies of donor-derived and intrinsic RPMs in mice adoptively transferred with fetal precursors (right). The presented data are representative of at least two independent experiments, except C and H, which come from a single experiment. Symbols in column graphs represent values of individuals and the column size mean (± SD). *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001 (unpaired two-tailed Student’s t test).

comparable to WT littermate controls (Fig. 3, C and D). Interestingly, VCAM-1 expression, which is critical for RPM development (Ulyanova et al., 2016), was substantially reduced in Pparg-deficient RPMs during the first week after birth (Fig. 3 E). Although Vav1-Cre/CreERT2/Pparg<sup>fl/fl</sup> mice lack PPARγ in all hematopoietic cells, the numbers of B cells, T cells, dendritic cells (DCs), and other myeloid subsets in spleen and blood were not changed in adult mice (Fig. S3, E and F), in support of a specific direct role of PPARγ for RPM differentiation.

Treatment of adult WT mice with the PPARγ antagonist (GW9662) or agonist (rosiglitazone) for 7 consecutive days had no effect on RPM number or their phenotype (Fig. 3 G). As IL-4 has been previously described as a potent activator of PPARγ expression in bone marrow-derived macrophages in vitro (Santo et al., 2010), we tested its capability to induce PPARγ in vivo. Overexpression of IL-4 by hydrodynamic gene delivery in WT mice led to an over fourfold increase in RPM number, an elevated frequency of Ki67<sup>+</sup> RPMs, and higher PPARγ mean fluorescence intensity (MFI) of RPM (Fig. 3 H). However, a complete deficiency of either IL-4 receptor or GM-CSF receptor (Cd2r, which are known inducers of PPARγ in M2 macrophages (Odegard et al., 2007; Santo et al., 2010) and AM (Bonfield et al., 2003; Schneider et al., 2014), respectively, did not cause any impairment of RPM development (Fig. 3 I).

The fetal liver contains progenitors with the capacity to give rise to multiple types of tissue macrophages (Epelman et al., 2014). To assess a cell-autonomous requirement of PPARγ for RPM development, we sorted CD45<sup>+</sup> cells from the fetal liver of E14.5 WT embryos (CD45.1) and injected them into newborn Vav1-Cre/CreERT2/Pparg<sup>fl/fl</sup> and Pparg<sup>−/−</sup> mice. 8 wk later, RPMs in Vav1-Cre/CreERT2/Pparg<sup>fl/fl</sup> mice were completely restored with cells derived from transferred WT fetal precursors (Fig. 3, J-L). Notably, transferred cells did not contribute to macrophages in the liver (Fig. S3, G and H). Overall, our data demonstrate an intrinsic requirement for PPARγ in the perinatal development of RPMs.

Impaired RPM development in the absence of PPARγ affects removal of senescent RBCs and reticulocytosis after hemolytic anemia

Although RPMs and EIMs were drastically reduced in the absence of PPARγ, we noted a small remaining population with comparable surface marker expression (Fig. 2, A and B). These Vav1-Cre/CreERT2/Pparg<sup>fl/fl</sup>-derived RPMs did not differ morphologically from their WT counterparts (Fig. 4 A), other than the fact that the cell size seems to be increased. To better characterize the transcriptional program regulated by PPARγ in RPMs and EIMs, we sorted the remaining F4/80<sup>+</sup>VCAM-1<sup>+</sup> population from the spleen and bone marrow of 7-d-old Vav1-Cre/CreERT2/Pparg<sup>fl/fl</sup> mice and compared their transcriptomes to Pparg-sufficient RPMs and EIMs from littermate controls using RNA sequencing. Principal component analysis (PCA) showed that the RPM and EIM molecular signatures differed between the two genotypes (Fig. 4 B). 485 and 377 DEGs were detected for RPMs and EIMs, respectively (with threshold |log2 ratio| > 1.5; FDR < 0.05), with the top 50 DEGs shown in Fig. 4 C and Fig. S4 A. GO analysis identified altered biological processes in Pparg-deficient cells, showing that pathways like chemotaxis or inflammatory/immune response were activated, while DNA replication, erythrocyte development, and heme biosynthesis were the most significantly reduced processes (Figs. 4 D and S4 B) in both subsets when compared with WT RPMs and EIMs. As is the case for Spic-deficient RPMs, the loss of PPARγ led to decreased expression of multiple RPM signature genes, while the expression of many genes from signatures of other tissue-resident macrophages was increased (Figs. 4 E and S4 C).

Vav1-Cre/CreERT2/Pparg<sup>fl/fl</sup> mice have a decreased number of EIMs, which are nurse cells for erythroblasts and control the proper development of reticulocytes (Bessis, 1958; Chow et al., 2013; Chow et al., 2011). Upon hemolytic stress and anemia induced by phenylhydrazine treatment (Fig. 4 F), Pparg-deficient mice (>20 wk old) showed an impaired release of reticulocytes into the blood stream (Fig. 4 G), as has been reported for mice deprived of CD16<sup>+</sup> macrophages (Chow et al., 2013); however, this difference was not observed in young Pparg-deficient mice (Fig. S4 G). Also, we did not observe a decrease in HCT for Vav1-Cre/Pparg<sup>−/−</sup> mice (Figs. 4 H and S4 H). Notably, the overall disposal of senescent RBCs was strikingly reduced in Vav1-Cre/CreERT2/Pparg<sup>−/−</sup> mice (Fig. 4, I and J), while Pparg-deficient RPMs possessed a comparable ability to phagocytose stressed RBC at the single cell level (Fig. 4 K; and Fig. S4, D–F). We propose that the strongly reduced number of RPMs in Vav1-Cre/Pparg<sup>−/−</sup> mice is the reason RBCs are cleared less efficiently. Moreover, Kupffer cells (KCs), which should help in degrading damaged RBCs, were not more active in Vav1-Cre/Pparg<sup>−/−</sup> mice (Figs. 4 K and S4 E); thus, it appears that a small number of RPM-like cells with the ability to phagocytose RBCs can develop independently of PPARγ, but this is not sufficient to allow for an efficient clearance of senescent RBCs and the reuse of iron.

PPARγ maintains RPM identity throughout life

Although PCA analysis showed that the transcriptomes of Spic- and Pparg-deficient RPMs differed significantly (Fig. 5 A), around 50 genes were commonly down- and upregulated for both KO’s (Fig. 5 B; threshold |log2 ratio| > 1.5). Csgal, Pf4, and Tox2 belong to the top three commonly upregulated genes, while Itgad, Vstm4, and Mrap include the top three commonly downregulated genes. Consistently, Csgal (CD88) surface levels were strikingly upregulated on RPMs and EIMs in the absence Pparg and Spic (Fig. 5, C and D). We next crossed Ubc-CreERT2, hereafter referred to as CreERT2, with Pparg<sup>−/−</sup> mice to generate CreERT2/Pparg<sup>−/−</sup> mice, allowing temporally controlled deletion of PPARγ. Tamoxifen treatment of adults resulted in the excision
Figure 4. PPARγ is not essential for iron metabolism and removal of senescent RBCs by RPMs. (A) Cytospins of sorted RPMs derived from adult Ppargfl/fl and Vav1-Cre/Ppargfl/fl mice. Black scale bars, 20 µM. (B) PCA performed on transcriptome from three biological replicates from Ppargfl/fl RPM (black dots), Vav1-Cre/Ppargfl/fl RPM (red dots), Ppargfl/fl EIM (green dots), and Vav1-Cre/Ppargfl/fl EIMs (blue dots). (C) Heat map showing top 50 DEGs between Ppargfl/fl and Vav1-Cre/Ppargfl/fl RPMs (according to absolute values of gfold). Cells were isolated from 7-d-old mice. (D) Upregulated (red) and downregulated (blue) GO terms for Biological Process (BP) from comparison of Vav1-Cre/Ppargfl/fl over Ppargfl/fl RPMs (P < 0.01). (E) Bar graphs showing upregulated (red) and downregulated (blue) genes from a comparison of Vav1-Cre/Ppargfl/fl over Ppargfl/fl RPMs (|log2 ratio| > 1.5) with gene signatures of different tissue-resident macrophages (MΦ) and monocytes derived from Lavin et al. (2014). (F) Graphical presentation of the experiment where hemolytic anemia was induced in Ppargfl/fl and Vav1-Cre/Ppargfl/fl mice (22–25 wk old); phenylhydrazine (PHZ; 30 mg/kg) was i.v. injected and blood samples were collected on indicated days. (G and H) Blood reticulocyte number (G) and HCT (H) was measured at indicated days. (I) Graphical presentation of the experiment where PKH26-labeled stressed RBCs were i.v. injected into Ppargfl/fl and Vav1-Cre/Ppargfl/fl mice (12–16 wk old); RPMs were stressed and labeled with the dye before injections and...
of floxed Pparg alleles and absence of PPARγ protein only in a fraction of RPMs and EIMs that can be separated from PPARγ-expressing cells by CD88 expression (Figs. 5 E and SS A). Indeed, CD88<sup>hi</sup> cells were observed only in tamoxifen-treated CreERT2/Pparg<sup>fl/fl</sup> mice, which confirms results obtained in Vav1-Cre/ Pparg<sup>fl/fl</sup> mice (Fig. 5, F and G) and suggests that PPARγ potently suppresses CD88 expression. Interestingly, absence of PPARγ did not affect RPM and EIM numbers 2.5 wk after the first tamoxifen treatment (Fig. 5 H), indicating that PPARγ is not required for their survival at steady state. Nonetheless, deletion of PPARγ in adult RPMs (i.e., sorted CD88<sup>hi</sup> RPMs) resulted in downregulation of 125 genes and upregulation of only 13 genes (|log<sub>2</sub> ratio| > 1.5; FDR < 0.05). The top 50 DEGs are listed in Fig. 5 I. Similar to Pparg-deficient RPMs and EIMs from young mice, GO analysis identified biological processes, like immune response and chemotaxis, as increased in tamoxifen-treated CreERT2/Pparg<sup>fl/fl</sup> cells (Fig. 5 J). However, the top pathways decreased in the previous comparisons in Vav1-Cre/Pparg<sup>fl/fl</sup> mice did not change significantly in CreERT2/Pparg<sup>fl/fl</sup> cells, and there were few changes in the expression macrophage signature genes associated with different tissue macrophage subsets (Fig. 5, J and K).

Taken together, these results suggest that PPARγ plays a pivotal role in proper localization and maturation of RPMs and EIMs, while its function in their maintenance at steady state is less critical.

Common transcriptional changes associated with Pparg and Spic deficiency in RPMs

The dependence of RPM development on PPARγ is reminiscent of the phenotype detected in adult Spic<sup>−/−</sup> mice. Given the decreased levels of PPARγ in RPMs from young Spic-deficient mice, we speculated that Spic-deficient RPMs could be similar to the RPMs detected in Vav1-Cre/Pparg<sup>fl/fl</sup> newborns. When we focused on similarities in the highly dysregulated genes (|log<sub>2</sub> ratio| > 2.5; FDR < 0.05) from Spic<sup>−/−</sup>, Vav1-Cre Pparg<sup>fl/fl</sup> RPMs and from adult tamoxifen-treated creERT2/Pparg<sup>fl/fl</sup> CD88<sup>hi</sup> RPMs, the shared upregulated genes included Csf1r, Itgt (integrin α<sub>1</sub>), and Cc3tr1, which are associated with the phenotype of patrolling monocytes (Fig. 6 A). Among the downregulated genes, 27 were shared between at least two sets (Fig. 6 B). The strongest gene reduction, common to all three cell subsets, was the gene encoding Itpad (integrin α<sub>0</sub>). We examined CD88 and CD11a, encoded by the Itgd gene, protein expression on WT RPMs at different time points (Fig. 6 C) and observed that both markers were downregulated at days 5 and 7 after birth in WTs but not in either KO samples (Fig. 6 D). In addition, both Spic- and Pparg-deficient RPMs displayed increased CD11b and decreased VCAM-1 expression levels, when compared with WT controls derived from age-matched littermates (Fig. 6 E).

Taken together, the data demonstrate that Spic and Pparg repressor expression of surface molecules (CD88) and orchestrate adhesion and migration of the RPM precursor by regulation of the integrins CD11b, CD11a, and CD11d.

Discussion

Recent studies have identified signature transcription factors important for the differentiation of a variety of cell types and have elucidated the mechanism by which environmental signals trigger their expression (A-Gonzalez et al., 2013; Abutbul et al., 2012; Buttgereit et al., 2016; Kierdorf et al., 2013; Okabe and Medzhitov, 2014; Schneider et al., 2014). To our knowledge, we are the first to describe the dependency of different tissue-resident macrophage subsets—iron-recycling macrophages and AMs—on the same transcription factor (PPARγ), even though the macrophages have seemingly different functions.

AMs start to develop perinatally from yolk sac–derived fetal liver monocytes (Guilliams et al., 2013; Schneider et al., 2014). Sensing of GM-CSF produced mainly by alveolar epithelial cells induces PPARγ activation and consequent transcriptional changes of target genes that orchestrate terminal differentiation and functional specialization. Deletion of Pparγ at birth—induced by Cdlc-Cre—arrests AM development at the level of a dysfunctional pro-AM with defects in lipid metabolism (Schneider et al., 2014). The two other Pparγ-dependent macrophage populations reported here, splenic RPMs and bone marrow EIMs, share an iron-recycling function. RPMs derive from fetal liver monocytes and are long lived (Epelman et al., 2014). Their development from monocytes has been shown previously to depend on another transcription factor, Spi-C (Kohyama et al., 2009). Spi-C is constitutively repressed in monocytes by Bach1, but is induced by increasing levels of heme, an intermediate product of hemoglobin degradation. Heme binds to Bach1 and marks the transcriptional repressor for protosional degradation (Haldar et al., 2014). Interestingly, we show that Spi-C is dispensable for the RPM and EIM expansion that occurs in the first week after birth. Nonetheless, Spic<sup>−/−</sup> RPMs differ significantly from their WT counterparts at the transcriptomic level. The expression of genes related to iron metabolism, like heme oxygenase 1 (Hmox1), ferritin (Fth1, Ftil), and ferroportin (Slc40a1), is decreased, indicating compromised functionality of Spic<sup>−/−</sup> RPMs. Similarly, Hmox1<sup>−/−</sup> mice lose iron-recycling macrophage populations with time, because they are unable to break down cytotoxic heme, leading to elevated oxidative stress and cell death (Kovtunovyych et al., 2010). Interestingly, Pparg-deficient RPM-like cells from Vav1-Cre/ Pparg<sup>fl/fl</sup> mice are able to phagocytose RBCs but are impaired in support of normal iron recycling.

The lack of Spi-C or PPARγ expression leads to a similar phenotype in adult mice. Both Spic<sup>−/−</sup> and Vav1-Cre/Pparg<sup>fl/fl</sup> mice have strongly reduced RPM and EIM populations and accumulate iron in the spleen and bone marrow (not shown),
while their blood contains normal levels of erythrocytes. Also, the decrease in RPMs does not influence the cellular composition of the spleen, indicating that deletion of PPARγ in other CD45+ immune cells—that express Vav1-cre—did not change the cell composition and indirectly cause the loss of RPMs. To definitively exclude this possibility, we experimentally confirmed that PPARγ is required intrinsically for RPM development.

Global PPARγ deficiency in mice is embryonically lethal (Barak et al., 1999). Using the Cre-loxP system, PPARγ deficiency has been studied in various cell subtypes and was identified as a master regulator of adipocyte differentiation (Siersbaek et al., 2010). Studies addressing the importance of PPARγ in the immune system show that this nuclear receptor is essential for AM development upon GM-CSF activation (Schneider et al., 2014) and M2 macrophage polarization triggered by IL-4 stimulation (Odegaard et al., 2007; Szanto et al., 2010). PPARγ is highly expressed in AMs and splenic macrophages, but it is low in most other hematopoietic cells in the steady state (Gautier et al., 2012a). We show that PPARγ starts being expressed by RPMs during the first few days after birth and persists at high levels into adulthood. This increased expression within the first days of life correlates with an abrupt expansion of this subset and a marked defect in RPM numbers in the absence of PPARγ. IL-4 and GM-CSF are well-established drivers of PPARγ expression in M2 macrophages and AMs, respectively; however, both IL-4R- and GM-CSFR-deficient mice showed no reduction in the RPM and EIM population, demonstrating that these cytokines are dispensable for RPM and EIM development. Nonetheless, overexpression of IL-4 by hydrodynamic gene delivery led to an expansion of RPMs in WT mice. It remains to be investigated which factors activate PPARγ in the RPM and EIM niches in the red pulp and bone marrow and whether or not activation requires a PPARγ lipid ligand (Daniel et al., 2018).

Loss of PPARγ expression led to changes in the transcriptome of sorted RPMs and EIMs. GO analysis for DEGs in RPM/EIM-like cells from Vav1-Cre/Ppargfl/fl mice compared with WT counterparts revealed a more pro-inflammatory phenotype of Pparγ-deficient RPM-like and EIM-like cells as early as 7 d after birth. Biological processes, like chemotaxis and cell migration, were increased, while DNA repair and cell-cycle pathways were strongly reduced. The reduction in transcripts from genes involved in cell cycling was not reflected in a reduced Ki67or 5-ethynyl-2′-deoxyuridine (EdU) incorporation in Pparγ-deficient RPM-like cells from Vav1-Cre/Ppargfl/fl newborn mice. This result needs further investigation but could be an indication of increased cell death or altered cell-cycle times in Pparγ-deficient RPM-like cells. Recently, it was shown that large peritoneal macrophages lacking Bhlhe40 are reduced in number due to a proliferative defect, despite normal BrdU incorporation. This was explained by the accumulation of cells in G1 phase and similar numbers in S, G2, and M phases (Jarjour et al., 2019).

The expression of multiple RPM signature genes was reduced upon PPARγ deficiency, while expression of genes associated with signatures of other tissue-resident macrophage subsets was increased. Analysis of CreERT2/Ppargfl/fl CD88hi RPMs from tamoxifen-treated mice further confirmed the role of PPARγ in maintaining RPM identity.

Interestingly, Spic-deficient and Pparγ-deficient RPMs share many similarities, which may be explained by reduction of Pparγ-levels in the absence of Spi-C. Both PPARγ- and Spic-C-deficient RPM-like cells fail to downregulate CD11a and CD88 on both the transcriptional and protein levels. We identified another member of the integrin family, integrin α9 (CD11d, encoded by Itgad), as strongly downregulated in deficient RPMs. Importantly, integrin α9 expression is specific to RPMs and, according to the Immunological Genome Project (http://www.immgen.org), is not expressed in any other tissue-resident macrophage subset. Elevated levels of integrin α9 have been linked to increased cell adhesiveness and macrophage retention in vascular lesions (Aziz et al., 2017; Yakubenko et al., 2008). Interestingly, the Itgad promoter contains several predicted binding sites for PPARγ and could be a novel target of this transcription factor. The interaction of integrin VLA4 (α4β1) and VCAM-1 is important for erythroblastoid island formation (Sadahira et al., 1995). Mice lacking VCAM-1 show a reduced number of RPMs and EIMs (Ulyanova et al., 2016). Thus, impaired expression of VCAM-1 during the first week after birth can explain the defect in expansion and migration RPMs and probably also EIMs in the absence of PPARγ.
We propose that both Spi-C and PPARγ are upregulated when RPM precursors enter the neonatal spleen and that PPARγ expression is partially controlled by Spi-C. Together, they induce upregulation of VCAM-1 and changes in expression of the integrins CD11a (going down) and CD11d (going up), thereby facilitating the docking of progenitors in the niche and allowing further differentiation. Upon loss of either of the two factors, the potential progenitor cannot be efficiently retained in the tissue and possibly migrates out or dies due to displacement and/or lack of a niche factor. Neonatal expansion and the delayed death of Spic-deficient RPMs compared with Pparg-deficient RPMs may be explained by a compensatory mechanism activated in the absence of Spi-C. Collectively, the presented data unequivocally demonstrate PPARγ as a central regulator of iron-recycling macrophage development. The program triggered in the spleen and bone marrow seems to be very distinct from the program induced by the same factor in AM differentiation and includes novel pathways not related to lipid metabolism, possibly due to the collaborative action of Spi-C and PPARγ on tissue-specific target gene expression.

Materials and methods
Mice
Pparg<sup>fl/fl</sup>(Pparg<sup>+/−</sup>) mice (Imai et al., 2004), kindly provided by P. Chambon (University of Strasbourg, Strasbourg, France), were backcrossed for eight generations to C57BL/6 before crossing to either LysM-Cre<sup>+/+</sup> (LysM-Cre) mice (Clausen et al., 1999), Tg(Itgax-cre)1-1Reiz (Cd11c-Cre) mice (Caton et al., 2007), B6.Cg-Tg(Vav1-Cre)A2Kio/J (Vav1-Cre) mice (de Boer et al., 2003), or B6.Cg-Ndor1Tg(UBC-cre/ERT2)1Ejb/2J (CreERT2) (Ruzankina et al., 2007), resulting in mice with PPARγ deficiency either in myeloid cells (LysM-Cre/Pparg<sup>fl/fl</sup>), CD11c+ cells...
(Cd11c-Cre/Ppargfl/fl), hematopoietic cells (Vav1-Cre/Ppargfl/fl), or all cells upon tamoxifen treatment (CreERT2/Ppargfl/fl). For deletion of the Pparg gene in CreERT2/Ppargfl/fl mice, 8-wk-old mice were injected i.p. with 2 mg tamoxifen (Sigma-Aldrich) every other day six times and analyzed 4 d later. The large majority of experiments were performed with littermate Ppargfl/fl and Vav1-Cre/Ppargfl/fl or CreERT2/Ppargfl/fl mice. Gt(Rosa)26Sor-mtII (Rosa26-RFP) mice, which contain Cre-inducible tandem dimer RFP (Luche et al., 2007), were crossed with Vav1-Cre in order to check the Cre recombination activity. Spic−/− mice (Kohyama et al., 2009) were backcrossed for four generations to C57BL/6 (except Fig. SI D, six-generation backcross littermates). All mice were housed in individually ventilated cages under specific pathogen-free conditions at the Swiss Federal Institute of Technology Phenomics Center (Zurich, Switzerland) and were used for experiments between 8 and 17 wk of age, unless otherwise stated. Animal experiments were approved by the local animal ethics committee (Cantonal Veterinary Office, Zurich, Switzerland) and were performed according to local guidelines (Swiss Animal Protection Ordinance, Zurich, Switzerland) and Swiss animal protection law.

Cell suspensions
Mice were killed by CO2 overdose. Organs were removed and cut into small pieces, and then digested and passed through a 70-µm cell strainer. Spleens and fetal liver were digested for 30 min at 37°C with 2 mg/ml of type IV collagenase (Worthington Biochemical) and 50 U/ml DNase I (Sigma-Aldrich). Adult livers were digested for 45 min at 37°C with 2 mg/ml of type IV collagenase and 50 U/ml DNase I. Liver cell suspensions were centrifuged at 20 g for 5 min, and the supernatants with reduced number of hepatocytes were resuspended in 30% Percoll (GE Healthcare) before density centrifugation at 2,000 rpm for 5 min, with low acceleration and no brake. Bone marrow was flushed from the femur and tibia of mice and passed through a 70-µm cell strainer. Erythrocytes were lysed with ammonium chloride–potassium bicarbonate buffer.

Iron staining
Deposits of ferric iron were visualized by histochemistry using Prussian Blue stain of 4-µm sections of formalin-fixed, paraffin-embedded tissues. Multiple images of each biological replicate were taken on areas outside of the segmented pixels to provide the integrated intensity of the segmented pixels in Blue to select blue pixels. Particulate Analyz (v1.50f) was used to quantify the area and the integrated intensity of the segmented pixels. Rug plots were generated using the Seaborn.distplot function in Python.

Immunohistochemistry
Isolated spleens were fixed overnight in freshly prepared 4% paraformaldehyde (Merck Millipore) at 4°C under constant agitation. Fixed spleens were embedded in 4% agarose and cut with a vibrotome (Leica VT-1200). 40-µm sections were blocked with PBS supplemented with 10% FCS, 1 mg/ml anti-FcyR (BD Biosciences), and 0.1% Triton X-100 (Sigma-Aldrich) for 30 min at room temperature. Next, a primary antibody mix containing anti-B220, anti-F4/80, and anti-metallophilic macrophage marker (CD169) was added and samples were incubated in 4°C overnight. After washing with PBS supplemented with 2% FCS and 0.1% Triton X-100, secondary antibodies were applied for 1 h at room temperature. Stained sections were transferred onto slides and coverslips were mounted with Dako fluorescent mounting media. Microscopic analysis was performed using a confocal microscope (Zeiss LSM 710), and images were processed with ZEN 2010 software (Carl Zeiss).

Cell proliferation in vivo
Pups were injected with EdU and splenocytes were analyzed 14 h later using Click-IT Plus EdU Alexa Fluor 488 Flow Cytometry Assay Kit (Thermo Fisher Scientific) according to the manufacturer’s instructions.

Flow cytometry
Multiparameter analysis was performed with LSR Fortessa (BD Biosciences), followed by data analysis with FlowJo software (TreeStar). All fluorochrome-conjugated or biotinylated monoclonal antibodies used are listed in Table 1. Dead cells were gated out using the viability marker eFluor780 (eBioscience). FcyIII/II receptors were blocked by incubation with anti-CD16/32 (2.4G2) purified from hybridoma supernatant (Swiss Federal Institute of Technology) before staining. PPARγ intracellular staining was performed using the Foxp3/Transcription Factor Staining Buffer Set (Thermo Fisher Scientific) according to the manufacturer’s protocol.

Cell sorting
Fetal liver cells were enriched for CD45+ cells using magnetic cell sorting (MACS) LS columns and anti-CD45 beads (both Miltenyi Biotec). Briefly, single-cell suspensions were incubated with 10 µl of beads/107 cells for 15 min at 4°C, followed by a wash with MACS buffer (PBS containing 1% BSA and 2 mM EDTA; both from Sigma-Aldrich). Pelleted cells were resuspended in 500 µl MACS buffer and loaded on equilibrated LS columns. Samples were washed twice with gravity flow of 4 ml MACS buffer and, after removing columns from the magnetic field, the purified fraction was eluted. CD45+ cells were later used for injections in Vav1-Cre/Ppargfl/fl newborns in 10 µl PBS (0.6 × 105/newborn). Fluorescence-activated cell sorting of RPMs/EIMs for cytospins, RBC phagocytosis, and RNA sequencing analysis was performed with a FACSAria IIIu (BD Biosciences).

RBC phagocytosis assay
In vivo
RBCs from whole blood were prepared as previously described (Theurl et al., 2016). In short, cells were washed with three volumes of PBS three times and centrifuged at 400 g for 10 min and subsequently stressed by incubation at 48°C for 20 min under continuous shaking. Next, stressed RBCs were resuspended in 1 ml of Diluent C (Sigma-Aldrich) and incubated...
for 5 min at room temperature with an equal volume of 4 µM PKH26 (Sigma-Aldrich) in the dark. Unbound particles were quenched with 2 ml of 100% FCS and the sample was spun down (400 g, 10 min) and washed two times with quenching buffer (PBS with 10% FCS). Finally, RBCs were resuspended in PBS and ~0.8 × 10⁹ RBCs were injected into the tail vein of Vav1-Cre/Ppargfl/fl and control mice. Some of the mice were sacrificed 16 h after injection and spleens and livers were isolated for single-cell suspension preparation. Blood samples were taken at 20 min, 16 h, 64 h, 88 h, and 90 h after injection.

In vitro
Sorted RPMs were plated on round glass coverslips in a 24-well plate containing RPMI 1640 plus GlutaMAX, 10% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin, and 50 µM β-mercaptoethanol (all Gibco) and left overnight to attach. The next day, RBCs were isolated from 200 µl of whole blood by several centrifugations with isotonic buffer (0.9% NaCl, 5 mM Na₃PO₄, pH 8) at low speed (600 g, 10 min). Cells were then lysed with 1 ml of hypotonic buffer (5 mM Na₃PO₄, pH 8) and spun down for 10 min at 12,000 g. Lysis and centrifugation were repeated until the pellet was white. Ghost RBCs were resuspended in 500 µl PBS + 0.1% BSA and incubated for 10 min at 37°C with an equal volume of 10 µM CFSE (Life Technologies). Unbound particles were quenched with 1 ml PBS containing 10% FCS and the sample was spun down (12,000 g, 10 min) and washed twice with quenching buffer. Finally, RBCs were resuspended in the same medium as RPMs and incubated with cultured cells for 1 h at 37°C. Afterwards, cells were washed twice with PBS to remove unbound RBCs and fixed with 4% paraformaldehyde (Sigma-Aldrich) for 15 min at room temperature. Glass coverslips were then washed with PBS and cells were perforated with PBS containing 10% FCS and 0.1% Triton X-100 for 1 h at room temperature. Next, cells were stained with FluorProbes 647-conjugated phalloidin (Interchim) for 1 h at room temperature. After extensive washing with PBS, cells were stained with DAPI (Sigma-Aldrich) and mounted on slides with Dako fluorescent mounting media. Samples were analyzed with Zen 2 software (Zeiss) on a fluorescence microscope (Zeiss AxioImager M2).

Quantitative real-time PCR
RNA was isolated with TRIzol reagent (Invitrogen) and reverse transcribed with GoScript RT (Promega) according to the manufacturer’s instructions. The resulting cDNA was used for quantitative real-time PCR with KAPA SYBR FAST (Sigma-Aldrich) performed on an i-Cycler (Bio-Rad). Expression was normalized to the housekeeping gene G6PDX expression, and the values were calculated using the comparative threshold cycle method (2⁻ΔΔCt). Primer sequences: G6PDX forward primer: 5'-CTACAGGTTCAGATGATGTC-3', reverse primer: 5'-CCCTTCTCCATTG-3'; and Pparg forward primer: 5'-GTGTAGTGGAAGACACTCGCAATG-3', reverse primer: 5'-CCATGAGGGAATGTTAGAAGCAC-3'.

RNA preparation and sequencing
1,000 cells (RPMs/EIMs) were sorted directly into the lysis buffer (0.2% Triton X-100, 2 U/µl RNase inhibitor [New England Biolabs] in RNase-free water [Thermo Fisher Scientific]). RNA isolation and sequencing were performed as described before (Picelli et al., 2014). Briefly, oligo-dT primers and dNTPs were added to lysed cells and hybridization of the primer to poly(A) tails of all mRNA was performed at 72°C for 3 min. Next, samples were reverse transcribed with LNA-containing TSO primer,
enabling template switching, and SuperScript II RT (Thermo Fisher Scientific) at 42°C for 90 min followed by 10 cycles of 2-min intervals at 50°C and 42°C in order to increase efficiency and complete the reaction. cDNA was preamplified using ISPCR primers containing the matching sequence to the outer fragments of oligo-DT and TSO primers. Amplification was performed with 18 cycles of 20 s denaturation at 98°C, 15 s annealing at 67°C, and 6 min extension at 72°C with KAPA HiFi HotStart ReadyMix (Kapa Biosystems). PCR products were purified with AMPure XP beads (Beckmann Coulter), and 1 ng of the product was tagmented using the Nextera XT DNA sample preparation kit (Illumina). Afterward, final enrichment with Index primers from the Nextera XT kit (Illumina) was performed for eight cycles of 30 s denaturation at 95°C, 30 s annealing at 55°C, and 30 s extension at 72°C, followed by purification with AMPure XP beads. Two nanomoles of each library were pooled for single-end DNA sequencing on a HiSeq4000 or NovaSeq instrument. Sequencing was performed at the Functional Genomics Center Zurich. The sequences were analyzed with the SUSHI application (Hatakeyama et al., 2016). In short, fragments were mapped to the Ensemble mouse reference genome GRCm38 (v25.06.2015) with the STAR aligner (Dobin et al., 2013). Values were then computed with the featureCounts function (Rsbread; Liao et al., 2013), and DEGs were detected with the Bioconductor package edgeR (Robinson et al., 2013). All statistical analyses were performed with Prism software (GraphPad). Comparisons of two groups were calculated with unpaired two-tailed Student’s t tests. Differences with a P value of <0.05 were considered significant.

Statistical analysis
All statistical analyses were performed with Prism software (GraphPad). Comparisons of two groups were calculated with unpaired two-tailed Student’s t tests. Differences with a P value of <0.05 were considered significant.

Online supplemental material
Fig. S1 depicts the gating strategy for identification of RPMs and numbers of RPMs and EIMs in Spic−/− and WT mice at different ages. Fig. S2 shows RPM and EIM numbers in Cdilc-Cre/Pparγfl/fl and LysM-Cre/Pparγfl/fl mice, as well as Cre activity in RPMs from Vavl-Cre/Rosa26-RFP mice. Fig. S3 shows RPM frequencies and numbers of lymphocytes and myeloid subsets in the spleen of Vavl-Cre/Pparγfl/fl mice. Fig. S4 shows differential gene expression in Pparγ-deficient EIMs; phagocytosis of RBCs; and stress erythropoiesis in Vavl-Cre/Pparγfl/fl mice. Fig. S5 shows spleen and bone marrow cellularity after conditional deletion of Pparγ in adults.

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Figure S1. Gating strategy for identification of RPMs and numbers of RPMs and EIMs in Spic+/+ and WT mice at different ages. (A) Gating strategy for identification of RPMs in splenocytes of Spic+/+ and Spic−/− mice harvested at day 7. (B and C) Identification of EIMs by flow cytometry in 10-wk-old (B) and 7-d-old (C) Spic+/+ and Spic−/− mice. Shown are dot plots from representative individuals of groups of mice pregated on live CD45+ single cells with adjacent bar graphs showing absolute numbers (top) and percentages (bottom) of EIMs. Symbols represent individual mice. (D) Bar graphs showing absolute numbers and percentages among CD45+ RPMs and EIMs in 2-wk-old and 4-wk-old Spic−/− mice. (E) Histogram showing PPARγ expression on F4/80+VCAM-1+ gated EIMs from a representative individual (left) and adjacent bar graph with values indicating MFI of individuals and mean ± SD of Spic+/+ and Spic−/− mice. The presented data are representative of two independent experiments. *, P < 0.05; **, P < 0.01; ***, P < 0.001 (unpaired two-tailed Student’s t test). FSC, forward scatter.
Figure S2. RPM and EIM numbers in Cd11c-Cre/Ppargfl/fl and LysM-Cre/Ppargfl/fl mice; Cre activity in RPMs from Vav1-Cre/Rosa26-RFP mice. (A) Flow cytometry of spleen and bone marrow from Ppargfl/fl, Cd11c-Cre/Ppargfl/fl, and LysM-Cre/Ppargfl/fl mice. Gated on live cells. (B and C) Total cell count of RPMs (B) and EIMs (C), gated as in A. (D) Cre activity in RPMs from Vav1-Cre/Rosa26-RFP mice. RPMs were gated as depicted on representative flow cytometry plots. Cre activity in RPMs is presented on the bar graph as RFP-positive cells. The presented data are representative of two independent experiments (A and D) or pooled from two independent experiments (B and C; mean and SD of two to three mice per group). No mark, not significant. ****, P < 0.0001 (unpaired two-tailed Student’s t test). FSC, forward scatter.
Figure S3. RPM frequencies and numbers of lymphocyte and myeloid subsets in the spleen of Vav1-Cre/Ppargfl/fl mice. (A) RPM frequencies of CD45+ cells at different days after birth. (B) Total cell counts of all splenic cells from Ppargfl/fl and Vav1-Cre/Ppargfl/fl mice at different days after birth. (C and D) Total Ly6C+ monocyte count and frequencies of CD45+ (D) at different days after birth. (E and F) Total counts and frequencies of the indicated cell subsets in the spleen (E) and blood (F), respectively, from Ppargfl/fl and Vav1-Cre/Ppargfl/fl mice at steady state. The indicated subsets were gated as live CD45+ cells: CD19+ (B cells), TCRβ+CD4+ and TCRβ+CD8+ T cells, CD11bhiLy6G+ (neutrophils), SiglecF+ (eosinophils), F4/80+CD11bhiVCAM-1+ (RPMs), MHCII+CD11c+CD8+ (CD8 DCs), MHCII+CD11c+CD11b+CD4+ or CD4− (CD11b DCs), and MHCII−CD11c−CD11bhiLy6C+ or Ly6C− (monocytes). (G) Dot plots showing KC origin in recipient mice. KCs were gated as live CD11bintF4/80+CD64+CD11cneg, and the red gates indicate CD45.1+ KCs (derived from transferred fetal progenitors). (H) KC counts supplemented with bar graph showing frequencies of donor-derived and intrinsic KCs for transferred mice. The presented data are from a single experiment (A–D, G, and H; mean and SD of two to five mice per group) or representative from three independent experiments (E and F; mean and SD of six mice per group). *, P < 0.05; **, P < 0.01; ***, P < 0.001 (unpaired two-tailed Student’s t test).
Figure S4. Differential gene expression in Pparg-deficient EIMs; phagocytosis of RBCs and stress erythropoiesis in Vav1-Cre/Ppargfl/fl mice. (A) Heat map showing the top 50 DEGs between Ppargfl/fl and Vav1-Cre/Ppargfl/fl EIMs (according to absolute values of gfold). Cells were isolated from 7-d-old mice. (B) Upregulated (red) and downregulated (blue) GO terms for Biological Process (BP) from comparison of Vav1-Cre/Ppargfl/fl over Ppargfl/fl EIMs (P < 0.01). (C) Bar graphs showing upregulated (red) and downregulated (blue) genes from a comparison of Vav1-Cre/Ppargfl/fl over Ppargfl/fl EIMs (|log2 ratio| > 1.5) within gene signatures of different tissue-resident macrophages (MΦ) and monocytes derived from Lavin et al. (2014). (D and E) Flow cytometry showing the uptake of RBC-PKH26–positive RBCs in the spleen (D) and liver (E) by RPMs and KCs (pseudotype as Lin−CD45+) 16 h after injection of PKH26-labeled stressed RBCs. (F) Representative photographs of sorted RPMs from Ppargfl/fl and Vav1-Cre/Ppargfl/fl mice, which were coincubated for 1 h with CFSE-labeled or unstained (US) lysed RBCs, followed by fixation and phalloidin staining. White scale bars, 20 µM. (G and H) Ppargfl/fl and Vav1-Cre/Ppargfl/fl mice (8 wk old) were injected i.v. with phenylhydrazine (PHZ; 75 mg/kg) and the count of reticulocytes in the blood (G) and HCT (H) were measured at the days indicated. The presented data are from a single experiment (A–C) or representative of two independent experiments (D–H). **, P < 0.01.
Figure S5. Spleen and bone marrow cellularity after conditional depletion of Pparg in adults. (A) Pparg expression determined by RT-PCR in RPMs derived from CD88lo and CD88hi from CreERT2/Ppargfl/fl mice treated with tamoxifen. The values are normalized to a house-keeping gene (G6PDX). (B and C) Cell counts of indicated populations in Ppargfl/fl and CreERT2/Ppargfl/fl mice in the spleen (B) and the bone marrow (BM; C). The presented data are representative of three independent experiments (A–C, mean and SD from three to five mice per group). *, P < 0.05; **, P < 0.01; ***, P < 0.001 (unpaired two-tailed Student’s t test).