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Fate Mapping Reveals Origins and Dynamics of Monocytes and Tissue Macrophages under Homeostasis

Simon Yona,[1,6,7] Ki-Wook Kim,[1,6,8] Yochai Wolf,[1,6] Alexander Mildner,[1] Diana Varol,[1] Michal Breker,[1] Dalit Strauss-Ayali,[1] Sergey Viukov,[2] Martin Guilliams,[3,9] Alexander Misharin,[4] David A. Hume,[5] Harris Perlman,[4] Bernard Malissen,[3] Elazar Zelzer,[2] and Steffen Jung[1,*]

[1]Department of Immunology
[2]Department of Molecular Genetics
The Weizmann Institute of Science, Rehovot 76100, Israel
[3]Centre d'Immunologie de Marseille-Luminy, Aix Marseille Université, INSERM U1104, CNRS UMR7280, 13288 Marseille Cedex 9, France
[4]Department of Medicine, Northwestern University, Chicago, IL 60611-3008, USA
[5]The Roslin Institute, University of Edinburgh, Midlothian EH25 9RG, UK
[6]These authors contributed equally to this work
[7]Present address: Division of Medicine, University College London, London WC1 6JJ, UK
[8]Present address: Department of Microbial Pathogenesis, Yale University School of Medicine, New Haven, CT 06510, USA
[9]Present address: Laboratory of Immunoregulation and Mucosal Immunology, Department for Molecular Biomedical Research, VIB, Ghent, Belgium
*Correspondence: s.jung@weizmann.ac.il
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SUMMARY

Mononuclear phagocytes, including monocytes, macrophages, and dendritic cells, contribute to tissue integrity as well as to innate and adaptive immune defense. Emerging evidence for labor division indicates that manipulation of these cells could bear therapeutic potential. However, specific ontogenies of individual populations and the overall functional organization of this cellular network are not well defined. Here we report a fate-mapping study of the murine monocyte and macrophage compartment taking advantage of constitutive and conditional CX_3CR1 promoter-driven Cre recombinase expression. We have demonstrated that major tissue-resident macrophage populations, including liver Kupffer cells and lung alveolar, splenic, and peritoneal macrophages, are established prior to birth and maintain themselves subsequently during adulthood independent of replenishment by blood monocytes. Furthermore, we have established that short-lived $Ly6C^+$ monocytes constitute obligatory steady-state precursors of blood-resident $Ly6C^-$ cells and that the abundance of $Ly6C^+$ blood monocytes dynamically controls the circulation lifespan of their progeny.

INTRODUCTION

The mononuclear phagocyte system (van Furth et al., 1972) today comprises monocytes, macrophages, and dendritic cells (DCs), as well as their respective committed bone marrow (BM)-resident progenitors (Geissmann et al., 2010). Collectively, these cells play central roles in the maintenance of tissue integrity during development and its restoration after injury, as well as the initiation and resolution of innate and adaptive immunity.

Historically, mononuclear phagocyte subpopulations have been defined according to anatomic location and surface marker profiles. More recently, this definition has been extended to comprise dependence of subpopulations on specific growth and transcription factors (Hashimoto et al., 2011; Lawrence and Natoli, 2011), subset-specific gene expression signatures (Robbins et al., 2008), and distinct ontogenies (Geissmann et al., 2010).

A major breakthrough in defining mononuclear phagocyte development in the adult organism was the identification of a clonotypic BM-resident founder cell, termed macrophage-DC precursor (MDP), that gives rise to peripheral mononuclear phagocytes while having lost granulocyte potential (Fogg et al., 2006). MDPs differentiate within the BM into monocytes (Varol et al., 2007) and dedicated DC precursors, the pre-DCs (Liu et al., 2009). Both of these cell types are subsequently released into the circulation to allow repopulation of peripheral tissue macrophages and classical Fms-like tyrosine kinase 3 ligand (Flt3L)-dependent DCs, respectively. The MDP-pre-DC route is critical to ensure the constant replenishment of the ephemeral DC compartment. The relative importance of local proliferation versus recruitment from monocytes in the maintenance of tissue macrophage homeostasis has been debated since the original definition of the mononuclear phagocyte system (Hume, 2006; Hume et al., 2002). The original studies of Kupffer cell turnover in the liver, for example, concluded that very few resident macrophages are actively in cycle (Crofton et al., 1978). This proliferation is ablated by glucocorticoids, which also removes blood monocytes. The authors concluded that the proliferating cells were monocyte derived. That conclusion was, however, undermined by later evidence that glucocorticoids oppose growth factor activity, including the macrophage growth factor CSF-1 (Hume and Gordon, 1984).

In certain situations, such as helminth-associated T helper 2 cell milieus, expansion of local macrophages can rely solely on proliferation of tissue-resident macrophages exclusive of monocyte influx (Jenkins et al., 2011). Under inflammatory conditions, resident macrophages are complemented by recruited monocytes that differentiate in situ into macrophages. However, when analyzed, the contribution of these infiltrating cells was found to be transient (Ajami et al., 2011; Leuschner et al., 2012), whereas resident macrophages bring about the resolution of the inflammatory response and restore macrophage homeostasis (Davies et al., 2011).

Myeloid cells, including mononuclear phagocytes, are known to arise from two successive hematopoetic waves, referred to as "primitive" and "definitive" (Orkin and Zon, 2008). As best exemplified for the brain microglia (Alliot et al., 1999; Ginhoux et al., 2010), mononuclear phagocytes can be generated during development from primitive macrophages generated in the yolk sac, an extraembryonic tissue. Once established, microglia cells maintain themselves throughout adult life by virtue of longevity and limited self-renewal without input from definitive hematopoesis (Ajami et al., 2007; Ginhoux et al., 2010; Mildner et al., 2007). Geissmann and coworkers recently provided evidence of the existence of a macrophage lineage that is independent of the transcription factor myb (Schulz et al., 2012). In addition to microglia, autonomy from definitive hematopoesis was shown for F4/80^{hi}CD11b^{int} tissue macrophage populations, including Kupffer and Langerhans' cells.

The chemokine receptor CX_3CR1 is widely expressed in the mononuclear phagocyte system. Indeed, mice harboring a GFP reporter in the CX_3CR1 locus (Jung et al., 2000) have been instrumental in identifying and defining MDPs (Fogg et al., 2006), pre-DCs (Liu et al., 2009), and tissue-resident mononuclear phagocyte populations (Bar-On et al., 2010; Lewis et al., 2011; Niess et al., 2005; Varol et al., 2009). Moreover, discrete expression of CX_3CR1/GFP in *Cx3cr1*^{gfp} animals led to the identification of two monocyte subsets in mice, characterized as $CX_3CR1^{int}Ly6C^+$ and $CX_3CR1^{hi}Ly6C^-$ cells (Geissmann et al., 2003; Palframan et al., 2001), corroborating earlier studies on human blood (Passlick et al., 1989). $CX_3CR1^{int}Ly6C^+$ monocytes are the correlate of human $CD14^+CD16^+$ and $CD14^+CD16^-$ monocytes (Cros et al., 2010) and poised to traffic to sites of infection and inflammation (Geissmann et al., 2003; Serbina et al., 2008). $CX_3CR1^{hi}Ly6C^-$ "patrolling" monocytes—the correlate of human $CD14^{lo}CD16^+$ cells (Cros et al., 2010)—have been shown to adhere and crawl along the luminal surface of endothelial cells (Auffray et al., 2007). In the absence of inflammation, $CX_3CR1^{int}Ly6C^+$ monocytes can return to the BM and differentiate into $CX_3CR1^{hi}Ly6C^-$ cells (Varol et al., 2007). However, it is unclear whether the generation of $CX_3CR1^{hi}Ly6C^-$ monocytes requires $CX_3CR1^{int}Ly6C^+$ monocytes as obligatory intermediate or whether $CX_3CR1^{hi}Ly6C^-$ monocytes can—like $CX_3CR1^{int}Ly6C^+$ monocytes (Varol et al., 2007)—also originate directly from MDPs. Moreover, if alternative pathways exist, their relative contribution to the steady-state generation of monocytes remains unclear.

Here, we report a fate mapping study of the murine mononuclear phagocyte compartment by using mice that harbor genes encoding conditional or constitutive active Cre recombinase in their CX_3CR1 loci. We have demonstrated that tissue-resident macrophage populations, including peritoneal, splenic, and lung macrophages, as well as liver Kupffer cells, are established prior to birth and in adult steady state disconnected from monocyte input. Side-by-side comparison of *Cx3cr1*^{cre} and *Cx3cr1*^{creER} mice crossed to reporter animals, as well as *Cx3cr1*^{gfp} mice, combined with bromodeoxyuridine (BrdU)-pulsing experiments, adoptive transfers, and the analysis of *Ccr2*-deficient mixed BM chimeras, provided critical insights into monocyte dynamics. Specifically, we have established that $CX_3CR1^{int}Ly6C^+$ monocytes form in steady state a short-lived obligatory precursor intermediate for the generation of $Ly6C^-$ monocytes and, presumably as CSF-1 sink, dynamically control the lifespan of their progeny.

RESULTS

Dual Origins of Resident Macrophages

To exploit the pronounced activity of the CX_3CR1 promoter for the study of the mononuclear phagocyte system, we manipulated the murine CX_3CR1 loci to harbor Cre recombinase genes. Specifically, we replaced the *Cx3cr1* gene with genes encoding either *cre* recombinase or a *cre* recombinase fusion to a mutant estrogen ligand-binding domain that requires the presence of the estrogen antagonist tamoxifen for activity (*Cre*ERT2) (Metzger et al., 1995), yielding *Cx3cr1*^{cre} and *Cx3cr1*^{creER} mice (Figure 1A).

Recent studies have highlighted the differential origins of discrete peripheral macrophage and DC compartments in adult mice (Geissmann et al., 2010). Given the broad expression of the *Cx3cr1* gene within the mononuclear phagocyte system, we decided to perform a side-by-side comparison of the reporter gene activation patterns in *Cx3cr1*^{gfp}, *Cx3cr1*^{cre}, and *Cx3cr1*^{creER} mice, the latter crossed to *R26-yfp* reporter animals (Figure S1 available online; Srinivas et al., 2001). Macrophage populations from *Cx3cr1*^{gfp} and *Cx3cr1*^{cre}*:R26-yfp* mice fell into two categories. Cells, such as microglia and intestinal and renal macrophages, express the CX_3CR1 chemokine receptor (Jung et al., 2000; Niess et al., 2005; Soos et al., 2006) and hence are found labeled in both *Cx3cr1*^{gfp} and *Cx3cr1*^{cre}*:R26-yfp* mice (Figure 1B). A second group of cells ceased to express the chemokine receptor but obviously originated from CX_3CR1^+ precursors, indicated by the absence and presence of label in *Cx3cr1*^{gfp} and *Cx3cr1*^{cre}*:R26-yfp* mice, respectively. This included peritoneal macrophages, liver Kupffer cells, and splenic and lung macrophages as well as epidermal Langerhans' cells (Figure 1C). Coexistence of the respective CX_3CR1-positive cells and CX_3CR1-negative cells that acquired the label during their past in steady-state tissues was confirmed by the histological examination of triple transgenic *Cx3cr1*^{gfp}*:Cx3cr1*^{cre}*:R26-rfp* mice, which harbored differentially labeled cells, as exemplified for RFP-positive CD11b^{int}F4/80^{hi} Kupffer cells and GFP-positive CD11b^{hi}F4/80^{int} perivascular macrophages in the liver (Figure 2Ai). Moreover, the notion that CX_3CR1-negative CD11b^{int}F4/80^{hi} Kupffer cells derive from cells that previously expressed CX_3CR1 was corroborated by the fact that CD11b^{int}F4/80^{hi} Kupffer cells were found to be GFP$^+$ in fetal livers but GFP$^-$ in adult livers of *Cx3cr1*^{gfp} mice (Figure 2Aii). Because in *Cx3cr1*^{cre}*:R26-yfp* mice Cre recombinase is constitutively active, CX_3CR1^+ precursors establish the label in

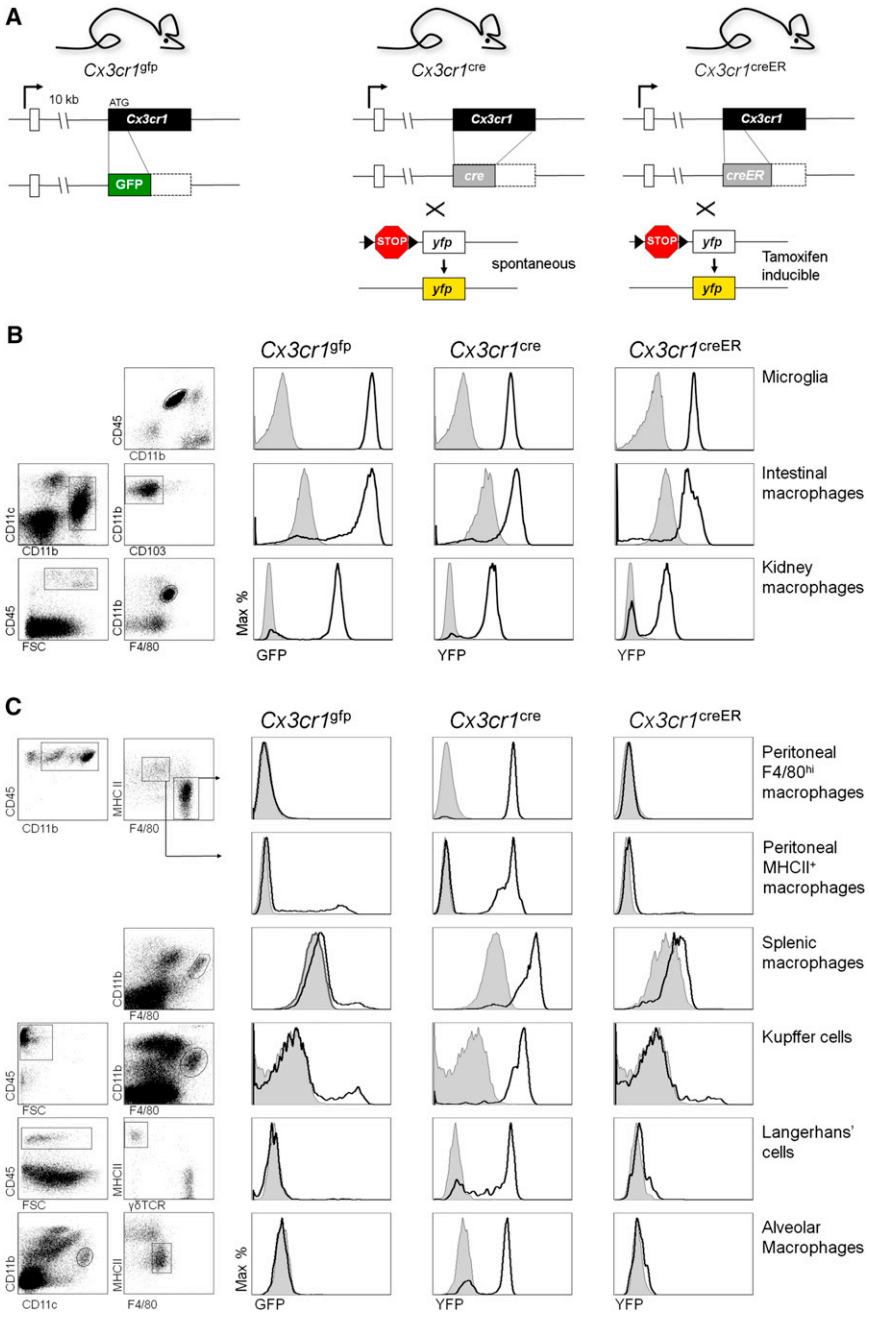


Figure 1. Reporter Gene Expression Profile of Macrophage Populations in *Cx3cr1*$^{gfp/+}$, *Cx3cr1*$^{cre/+}$*:R26-yfp*, and Tamoxifen-Treated *Cx3cr1*$^{creER/+}$*:R26-yfp* Mice

(A) Schematic of modified *Cx3cr1* loci. The *Cx3cr1*^{cre} and *Cx3cr1*^{creER} mice were crossed to *R26-yfp* mice, in which irreversible induction of YFP expression is induced upon *Cre* recombinase expression and activation, respectively.

(B and C) Flow cytometric analysis of CX_3CR1^+ (B) and CX_3CR1^- (C) mononuclear phagocyte populations of *Cx3cr1*$^{gfp/+}$, *Cx3cr1*$^{cre/+}$*:R26-yfp*, and *Cx3cr1*$^{creER/+}$*:R26-yfp* mice. *Cx3cr1*$^{creER/+}$*:R26-yfp* mice were treated for 4 weeks with tamoxifen prior to analysis. Results are representative of six mice per group.

CX_3CR1-negative tissue macrophages could be associated with prenatal development, or represent adult precursors, such as MDPs or monocytes (Fogg et al., 2006; Jung et al., 2000). To distinguish between these two scenarios, we took advantage of *Cx3cr1*^{creER}*:R26-yfp* mice, in which Cre activity can be regulated by drug administration and hence induced after birth. One month of continuous tamoxifen exposure of adult animals resulted in the efficient reporter gene expression in CX_3CR1^+ resident macrophages and microglia of *Cx3cr1*^{creER}*:R26-yfp* mice (Figure 1B). Moreover, it yielded substantial excision of the *LoxP*-flanked STOP cassette from the R26-STOP-YFP loci, and hence YFP expression, in the monocyte compartment (see below). However, no reporter gene expression was detected in the CX_3CR1^- macrophage populations of the tamoxifen-treated *Cx3cr1*^{creER}*:R26-yfp* mice, signifying that no monocytes had entered the pool.

To formally establish that descendants of monocytes from tamoxifen-treated animals can be detectable according to reporter gene expression after an extended period of time in a tissue context, we resorted to an adoptive transfer strategy focusing on intestinal resident macrophages that we previously established to be Ly6C$^+$ monocyte derived (Varol et al., 2009). Specifically, Ly6C$^+$ YFP$^-$ BM monocytes isolated from tamoxifen-treated mice were adoptively transferred into diphtheria toxin-treated CD11c-DTR→WT BM chimeras (Varol et al., 2009). As seen in Figure 2B, grafted cells gave rise to YFP$^+$ lamina propria macrophages. Notably, because the recipients were not treated with tamoxifen, the rearrangement of the R26-STOP-YFP loci must have occurred in the donor animals. The fact that monocyte-derived cells could be detected in tamoxifen-treated adult *Cx3cr1*^{creER}*:R26-yfp* mice according to their reporter expression was further corroborated by analyzing mice challenged intraperitoneally with thioglycollate, which led to the appearance of RFP$^+$ cells in the peritoneal cavities 72 hr after treatment (Figure 2C). Interestingly, after the resolution of this response, cells derived from the monocyte infiltrate persisted for up to 2 months after challenge. This was associated with a phenotypic shift to MHCII^{lo}F4/80^{hi} cells, suggesting the integration of the monocyte-derived cells into the resident macrophage pool (Figure 2C).

Collectively, the absence of label in resident CX_3CR1^- macrophage populations of tamoxifen-treated *Cx3cr1*^{creER}*:R26-yfp* mice establishes that there is no on-going steady-state

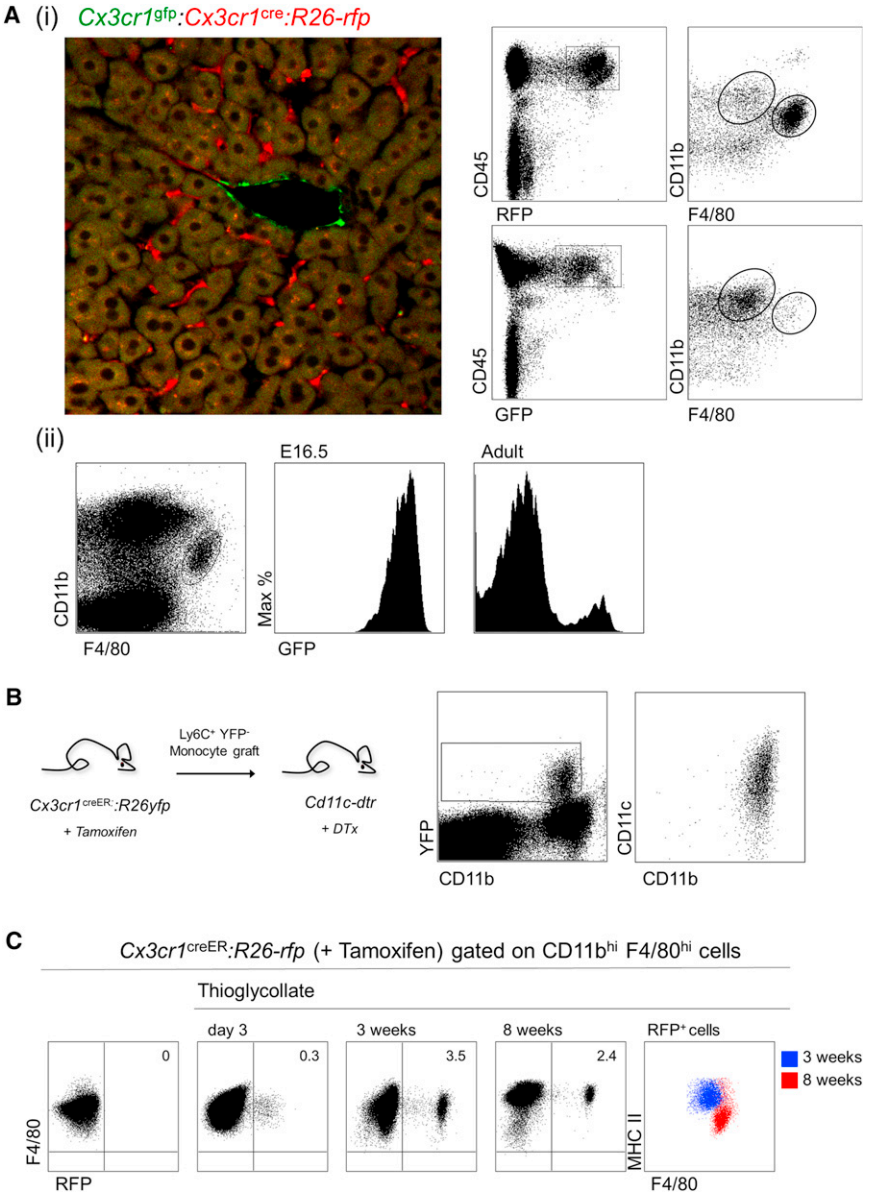


Figure 2. Dual Origins of Macrophages

(A) (i) Flow cytometric and histological analysis of livers obtained from *Cx3cr1*$^{cre/+}$*:R26-rfp:Cx3cr1*$^{gfp/+}$ mice. In these mice GFP expression acts as a direct reporter for CX_3CR1 expression, whereas RFP expression is controlled by *Cre* recombinase. (ii) Analysis of fetal and adult liver Kupffer cells of *Cx3cr1*$^{gfp/+}$ mice.

(B) Flow cytometric analysis of the intestinal lamina propria of DTx-treated *Cd11c-dtr* mice that received 7 days earlier a Ly6C$^+$YFP$^-$ monocyte graft isolated from tamoxifen-treated *Cx3cr1*$^{creER/+}$*:R26-yfp* mice. Results are representative of two experiments involving three mice per group.

(C) Flow cytometric analysis of peritoneal lavages of *Cx3cr1*$^{creER/+}$*:R26-rfp* mice administered a single tamoxifen gavage (5 mg) 1 day after an intraperitoneal thioglycollate injection. Rightmost graph shows phenotypic shift of monocyte-derived cells between 3 weeks and 8 weeks. Results are representative of two independent experiments involving three mice per group.

contribution of monocytes to Kupffer cells or peritoneal, splenic, and lung macrophages and that these cells derive prenatally originating either independent from the yolk sac or fetal liver cells and subsequently maintaining themselves through longevity and limited self-renewal.

Characterization of Myeloid Precursors and Monocytes in *Cx3cr1*^{cre}*:R26-yfp* and *Cx3cr1*^{creER}*:R26-yfp* Mice

CX_3CR1 chemokine receptor expression commences with the first dedicated mononuclear phagocyte precursor, the MDP, and expression is subsequently maintained in DC-committed precursors (CDPs, pre-DCs) and monocytes (Fogg et al., 2006; Liu et al., 2009; Onai et al., 2007). All these cell populations are consequently homogeneously marked by reporter gene expression in *Cx3cr1*^{gfp} mice (Figure 3A). Interestingly, however, reporter gene expression was absent from MDPs of *Cx3cr1*^{cre}*:R26-yfp* mice; rather, YFP$^+$ cells first appeared at the CDP and Ly6C$^+$ monocyte stage (Figure 3). This delay of YFP expression compared to *Cx3cr1*^{gfp} mice may be due to the time restraint imposed by the required rearrangement for STOP cassette excision prior to reporter gene expression in *Cx3cr1*^{cre}*:R26-yfp* animals. In *Cx3cr1*^{creER}*:R26-yfp* mice, where reporter activation requires the nuclear translocation of the CreER protein prior to rearrangement, YFP expression was found to be even further restricted. Thus, in tamoxifen-treated *Cx3cr1*^{creER}*:R26-yfp* mice, reporter gene expression was absent from CDPs and Ly6C$^+$ monocytes (Figure 3B). Only Ly6C$^-$ monocytes—and only a fraction of them—expressed YFP even after extended tamoxifen treatment (Figure 3B). The differential appearance of the YFP label in Ly6C$^+$ and Ly6C$^-$ monocyte subsets indicated distinct temporal requirements for their creation, supporting the notion of their sequential generation (Sunderkötter et al., 2004; Varol et al., 2007; Yrlid et al., 2006). Notably, key surface markers recently reported for the two monocytes subsets (Ingersoll et al., 2010) displayed gradual alterations between Ly6C$^+$YFP$^-$, Ly6C$^-$YFP$^-$, and Ly6C$^-$YFP$^+$ cells in *Cx3cr1*^{creER}*:R26-yfp* mice (Figure S2A). Furthermore, comparison of expression of these markers by *Cx3cr1*^{cre}*:R26-yfp* monocyte subsets also demonstrated gradual changes from Ly6C$^+$YFP$^-$ to Ly6C$^+$YFP$^+$ and Ly6C$^-$YFP$^+$ cells (Figure S2B). Taken together, reporter gene expression kinetics in *Cx3cr1*^{cre} and *Cx3cr1*^{creER}*:R26-yfp* mice and the gradual acquisition of maturation markers in the monocyte subsets support the notion that Ly6C$^+$ monocytes differentiate into Ly6C$^-$ monocytes in steady state.

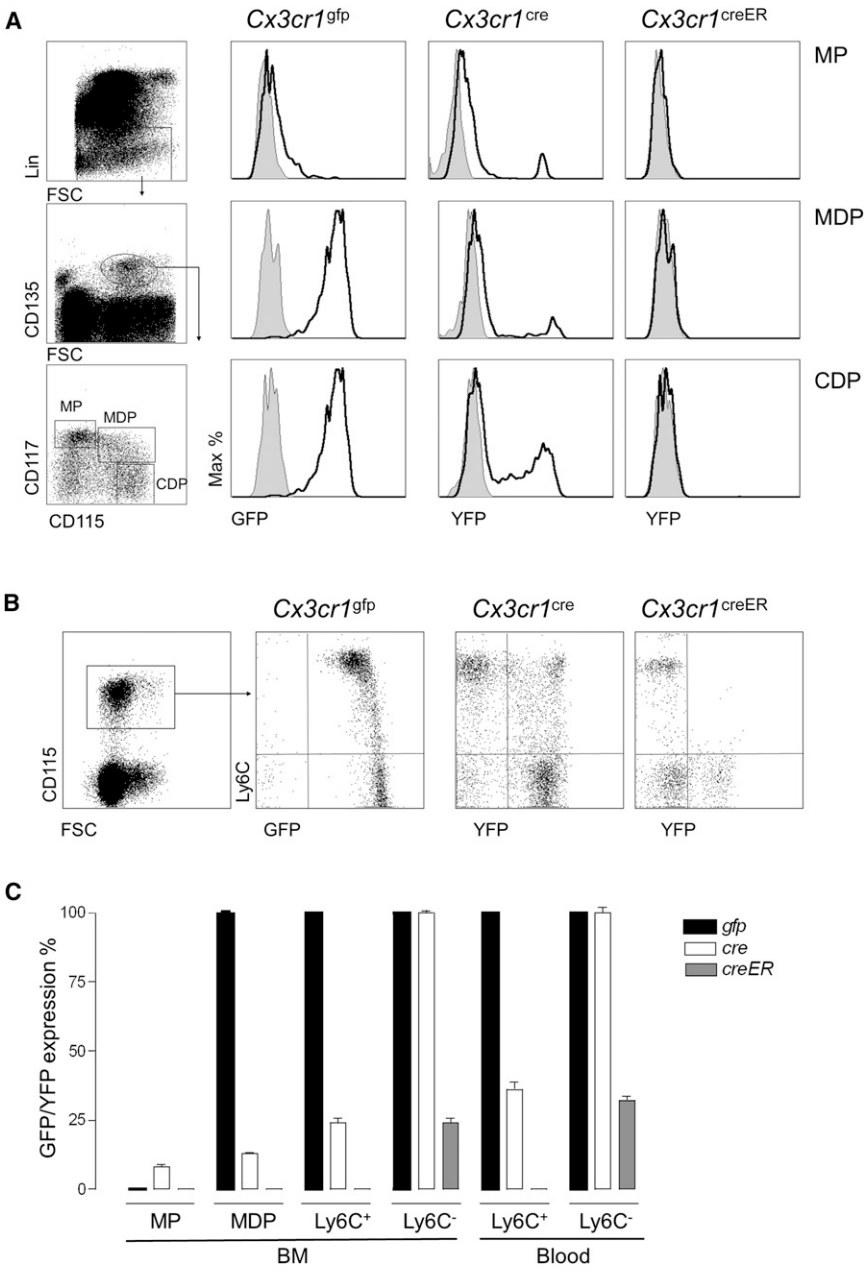


Figure 3. Reporter Gene Expression Profile of Mononuclear Phagocyte Precursors and Circulating Monocytes

(A and B) Flow cytometric analysis of different mononuclear phagocyte populations: precursors (A) and blood monocytes (B) obtained from *Cx3cr1*$^{gfp/+}$, *Cx3cr1*$^{cre/+}$*:R26-yfp*, and *Cx3cr1*$^{creER/+}$*:R26-yfp* mice. *Cx3cr1*$^{creER/+}$*:R26-yfp* mice were treated for 4 weeks with tamoxifen prior to analysis. Results are representative of four to six mice per group.

(C) Bar graph summarizing frequencies of GFP or YFP$^+$ cells in indicated mononuclear phagocyte populations of *Cx3cr1*$^{gfp/+}$, *Cx3cr1*$^{cre/+}$*:R26-yfp*, and *Cx3cr1*$^{creER/+}$*:R26-yfp* mice. Mean ± SEM are performed with four to six mice per group.

See also Figures S1 and S2.

ure S3A). Recipients were sacrificed at various time points after adoptive transfer and subjected to flow cytometry analysis. One day after transfer, grafted cells were detectable in recipient blood, BM, and spleen as Ly6C$^+$CX$_3$CR1/GFP^{int} cells (Figure S3B). However, corroborating our earlier study (Varol et al., 2007), by day 3 grafted splenic Ly6C$^+$ monocytes had quantitatively differentiated into Ly6C$^-$CX$_3$CR1/GFP^{hi} cells.

Next, we compared the differentiation potential of Ly6C$^+$YFP$^-$ and Ly6C$^+$YFP$^+$ monocytes of *Cx3cr1*^{cre}*:R26-yfp* mice. Isolated monocyte subsets were grafted into congenic WT recipient mice. Analysis over a 3 day period revealed a number of sequential steps required for Ly6C$^+$ monocytes to convert. Ly6C$^+$YFP$^-$ monocytes transformed from being YFP negative to cells expressing the YFP reporter prior to converting into Ly6C$^-$ monocytes, authenticating the notion that Ly6C$^+$ monocytes are the precursors of Ly6C$^-$ monocytes and our earlier assumption that acquisition of the YFP label by monocytes is a matter of time (Figure S4).

Ly6C$^-$ Monocytes Derive from Ly6C$^+$ Monocytes Independently in BM and Blood

With the definition of MDPs as in vivo monocyte precursors (Varol et al., 2007), the question arose whether these cells give rise to both CX$_3$CR1^{int}Ly6C$^+$ and CX$_3$CR1^{hi}Ly6C$^-$ monocytes or whether the two monocyte subsets are part of a developmental sequence (Sunderkötter et al., 2004; Varol et al., 2007; Yrlid et al., 2006). Although adoptively transferred Ly6C$^+$ CX$_3$CR1^{int} monocytes isolated from BM can give rise to Ly6C$^-$ CX$_3$CR1^{hi} monocytes in recipient mice (Varol et al., 2007), these grafts could have arguably been contaminated with immature BM precursors. To exclude this possibility, we isolated CX$_3$ CR1$^{GFP/+}$Ly6C$^+$ monocytes from the splenic reservoir (Swirski et al., 2009) and grafted them into congenic WT mice (Fig-

To further investigate the interrelation of blood monocytes and determine their turnover rates, we resorted to a 5-bromo-2′-deoxyuridine (BrdU) pulsing regime. Specifically, mice were dosed with three intraperitoneal (i.p.) injections of 2 mg BrdU (3 hr apart) and subsequently monitored over a 5 day period for the presence of the thymidine analog in the genome of the monocyte subsets. This low dose of BrdU is unlikely to be mitogenic (Takizawa et al., 2011). Because only a minor subset of monocytic cells is in cell cycle, BrdU is expected to be incorporated into dividing monocyte precursors, such as MDPs. One day after the first BrdU injection, all circulating Ly6C$^+$ monocytes were BrdU positive (Figure 4A). In contrast, no Ly6C$^-$ blood monocytes had incorporated label by day 1, indicating that these cells are not recently generated from a dividing precursor. The

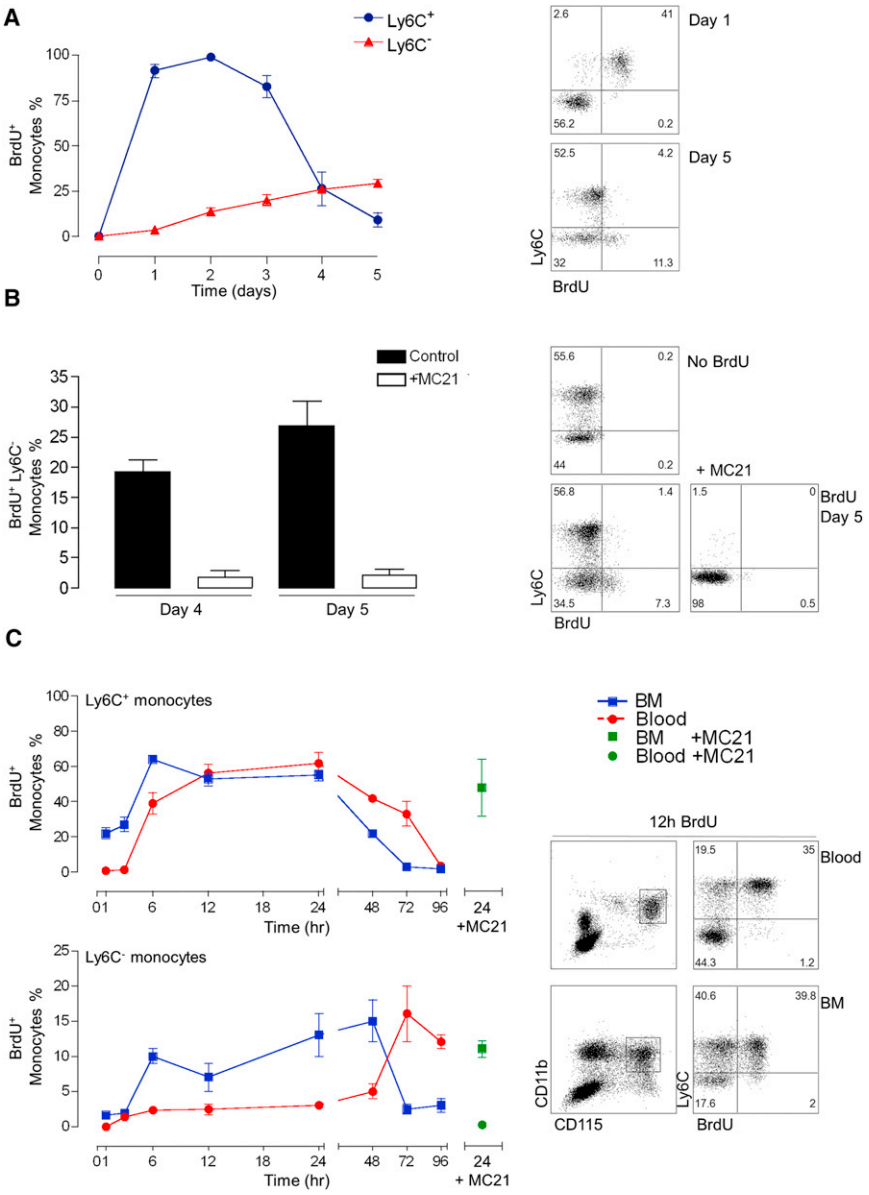


Figure 4. Monocyte Subset Dynamics

(A and B) Flow cytometric analysis of Ly6C$^+$ and Ly6C$^-$ blood monocyte subsets pulsed three times with 2 mg BrdU 3 hr apart and monitored over 5 days. (B) Mice were treated with CCR2 antibody MC21 and BrdU incorporation by Ly6C$^-$ blood monocyte was analyzed.

(C) Analysis of time course of BrdU incorporation of Ly6C$^+$ and Ly6C$^-$ monocytes in blood (red) and BM (blue) after a single 2 mg pulse of BrdU; BrdU incorporation by monocytes in mice treated with the MC21 antibody (green). Mean ± SEM are performed with three to four mice per group.

See also Figures S2–S6.

fraction of BrdU$^+$ cells in the Ly6C$^-$ compartment gradually increased to around one-third by day 5 (Figure 4A), whereas the Ly6C$^+$ monocytes lost the label by day 5. Fitting an exponential trendline from day 2 (i.e., the peak of BrdU label) to day 5, we calculated the steady-state half-life of the Ly6C$^+$ blood monocytes to be 0.843 days (20 hr) (r^2 = 0.932). The sequential acquisition of the BrdU label by the blood monocyte subsets suggested that it was incorporated into monocyte precursors that differentiated into Ly6C$^+$ monocytes and subsequently moved with time to Ly6C$^-$ monocytes. To corroborate this assumption, we took advantage of a cell ablation strategy targeting Ly6C$^+$ monocytes based on the injection of the CCR2 antibody MC21 (Brühl et al., 2007). MC21 treatment of BrdU-pulsed mice on day 1 efficiently ablated CCR2$^+$Ly6C$^+$ monocytes and spared as reported CCR2$^-$Ly6C$^-$ cells (Figure 4B). Interesting though and supporting the notion of a precursor/product relationship of the two monocyte subsets, removal of BrdU-labeled Ly6C$^+$ monocytes also prevented accumulation of the BrdU label in the Ly6C$^-$ monocyte population (Figure 4B).

To refine our analysis and extend it to monocytes residing in BM, C57BL/6 animals were injected with a single dose of BrdU (2 mg) (Figure 4C). Analysis of blood monocytes confirmed the sequential appearance of the BrdU label in Ly6C$^+$ and Ly6C$^-$ monocytes, respectively. Ly6C$^+$ monocytes in the BM were labeled before Ly6C$^+$ blood monocytes, consistent with their generation from MDPs in the BM (Varol et al., 2007). BM-resident Ly6C$^-$ cells were found to incorporate BrdU well before the blood Ly6C$^-$ monocytes, though still delayed when compared to BM Ly6C$^+$ monocytes. This suggests that Ly6C$^-$ BM monocytes are generated in the BM and Ly6C$^-$ blood monocytes are generated in the blood. In line with this notion, MC21 treatment that ablates Ly6C$^+$ blood but not Ly6C$^+$ BM monocytes did not impair labeling of BM Ly6C$^-$ monocytes (Figure 4C).

Collectively, these data establish that Ly6C$^+$ monocytes are precursors of Ly6C$^-$ cells. Moreover, the limited acquisition of BrdU label in the Ly6C$^-$ monocytes supports the earlier notion based on adoptive transfer experiments (Geissmann et al., 2003) that these cells have an extended circulation half life as compared to Ly6C$^+$ monocytes. Finally, differential label acquisition by Ly6C$^-$ BM and blood monocytes suggest that these compartments are established, and in steady state maintained, independent of each other.

Ly6C$^+$ Blood Monocytes Are Obligatory Precursors of Steady-State Ly6C$^-$ Monocytes

Among monocytes, membrane expression of the chemokine receptor CCR2 is restricted to Ly6C$^+$ cells and a prerequisite for these cells to exit from the BM (Serbina and Pamer, 2006; Shi et al., 2011). Accordingly, Ly6C$^+$ monocytes are

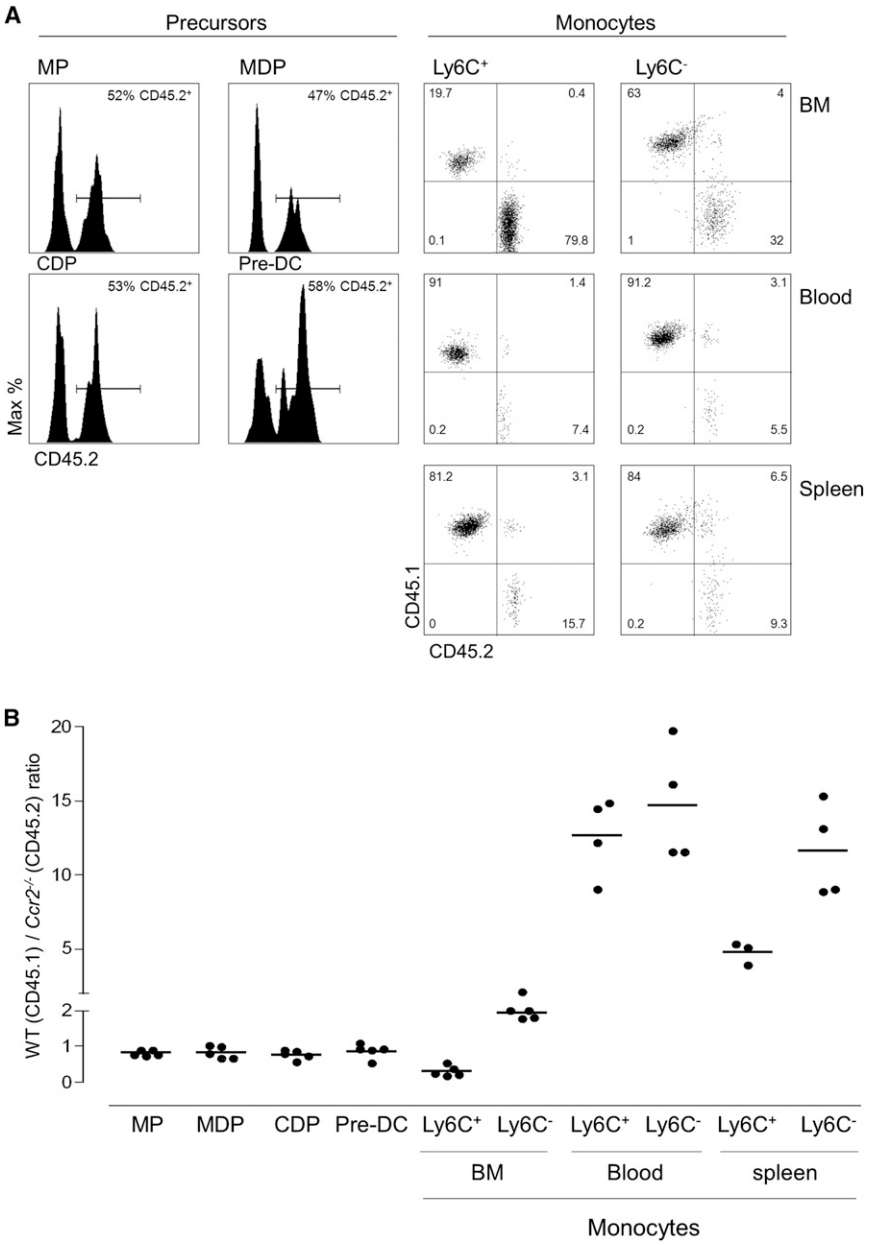


Figure 5. Impaired BM Exit of Ly6C$^+$ Blood Monocyte Affects the Ly6C$^-$ Cell Compartment

(A) Flow cytometric analysis of mixed *Ccr2*$^{-/-}$*:Cx3cr1*$^{gfp/+}$ (CD45.2)/*Ccr2*$^{+/+}$*:Cx3cr1*$^{gfp/+}$ (CD45.1) → WT BM chimeras. Distribution of cells within the mononuclear compartment were analyzed for percentage of CD45.2$^+$ cells. Cell definitions for gating: MP, Lin$^-$CD135$^+$CD115$^-$CD117$^+$; MDP, Lin$^-$CD135$^+$CD115$^+$CD117$^+$; CDP, Lin$^-$CD135$^+$CD115$^+$CD117$^-$; pre-DC, Lin$^-$CD11c^{int}MHCII$^-$CD135$^+$CD172a$^-$CX$_3$CR1$^+$; monocyte, CD11b$^+$ CD115$^+$. Results are representative of six analyzed mice.

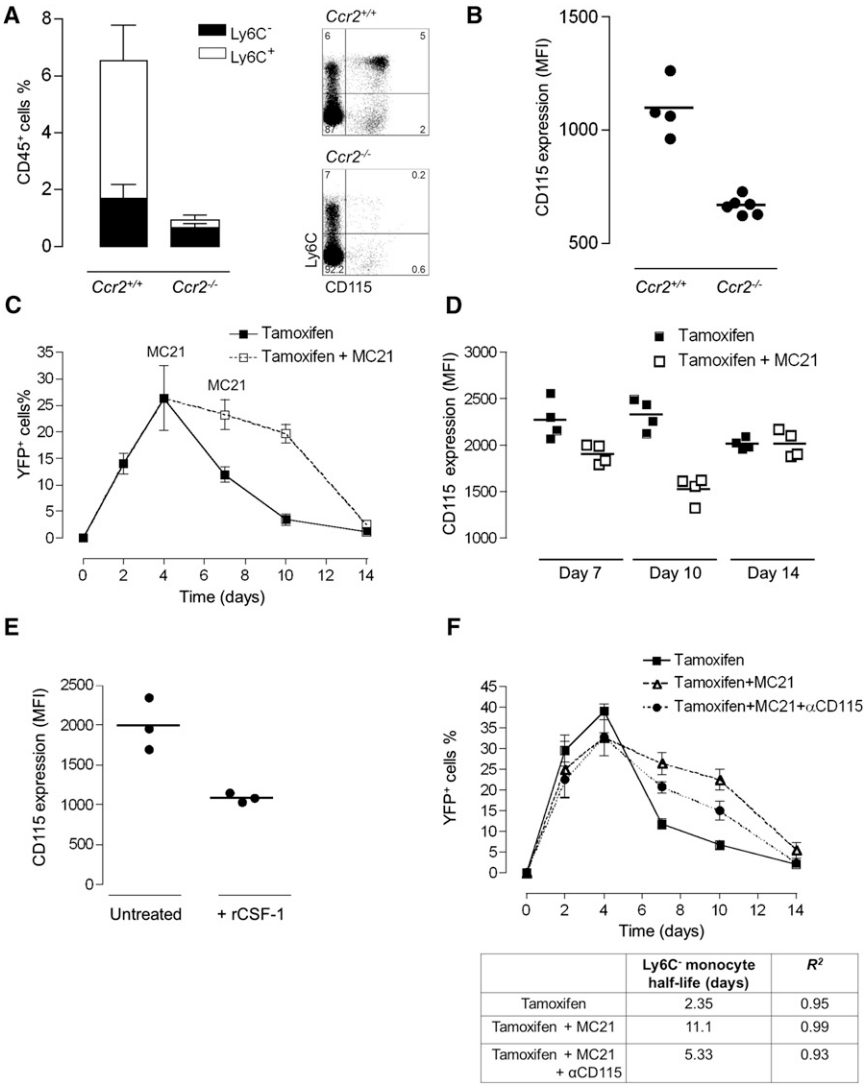
(B) Analysis of mixed *Ccr2*$^{-/-}$*:Cx3cr1*$^{gfp/+}$ *(cd45.2)/Ccr2*$^{+/+}$*:Cx3cr1*$^{gfp/+}$ (CD45.1) → WT BM chimeras. Representative result of two experiments involving three to five mice per group. See also Figures S3–S6.

reported to be selectively reduced in the circulation of CCR2-deficient mice (Serbina and Pamer, 2006). If Ly6C$^+$ blood monocytes are precursors of Ly6C$^-$ monocytes, the impairment of Ly6C$^+$ cell compartment would be expected to impinge on Ly6C$^-$ monocytes. To probe this point more rigorously, we analyzed monocyte subset distributions in a competitive setting by generating mixed chimeras, with BM isolated from CCR2-proficient (CD45.1) and CCR2-deficient (CD45.2) *Cx3cr1*$^{gfp/+}$ mice. Analysis of the BM of the respective chimeras 8 weeks after irradiation revealed that the two genotypes were equally prevalent among MPs and MDPs, as well as DC precursors (CDPs and pre-DCs) (Figures 5A and 5B). BM-resident CCR2 mutant Ly6C$^+$ monocytes were found to accumulate, presumably as a result of their impaired BM exit (Serbina and Pamer, 2006), whereas BM-resident mutant Ly6C$^-$ monocytes were moderately dominated by CD45.1$^+$ WT cells (Figure 5B). As expected from the reported phenotype of *Ccr2*$^{-/-}$ mice (Serbina and Pamer, 2006), in the blood Ly6C$^+$*Ccr2*$^{-/-}$ monocytes (CD45.2) were outcompeted by their CD45.1$^+$ WT counterpart (Figure 5A), but CCR2 deficiency also compromised the Ly6C$^-$ blood monocytes, with mutant cells underrepresented in both compartments (Figures 5A and 5B). The competitive disadvantage of both Ly6C$^+$ and Ly6C$^-$ CCR2-deficient monocytes was also evident in the spleen (Figure 5B), although it was less pronounced for Ly6C$^+$ cells, which after having entered the splenic pool might be trapped because of impaired mobilization.

Collectively, this establishes that the generation of Ly6C$^-$CX$_3$CR1^{hi} monocytes in steady state critically depends on CCR2-proficient Ly6C$^+$CX$_3$CR1^{int} monocytes as immediate precursors. Moreover, in order to serve as Ly6C$^-$CX$_3$CR1^{hi} monocyte progeny, these cells have to exit from the BM to the circulation.

Ly6C$^+$ Blood Monocytes Negatively Control the Lifespan of Ly6C$^-$ Monocytes

The fact that *Ccr2*$^{-/-}$ mice harbor circulating Ly6C$^-$CX$_3$CR1^{hi} monocytes despite their impaired Ly6C$^+$ monocyte compartment (Qu et al., 2004) seemingly contradicts the notion that the latter cells require Ly6C$^+$ monocytes as precursors. Indeed, analysis of the frequencies of the two monocyte subsets in *Ccr2*$^{-/-}$*Cx3cr1*^{gfp} mice confirmed the presence of a sizable Ly6C$^-$CX$_3$CR1^{hi} monocyte population, albeit drastically reduced as compared to *Ccr2*$^{+/+}$*:Cx3cr1*^{gfp} littermates (Figure 6A). Interestingly, Ly6C$^-$ blood monocytes of *Ccr2*$^{-/-}$ mice had downmodulated their receptors for the macrophage growth factor CSF-1,



	Ly6C- monocyte half-life (days)	R^2
Tamoxifen	2.35	0.95
Tamoxifen + MC21	11.1	0.99
Tamoxifen + MC21 + αCD115	5.33	0.93

Figure 6. Prevalence of Ly6C$^+$ Blood Monocytes Determines the Circulation Half-Life of Ly6C$^-$ Blood Cells

(A) Analysis of Ly6C$^+$ and Ly6C$^-$ blood monocyte subsets of *Ccr2*$^{+/+}$*Cx3cr1*$^{gfp/+}$ and *Ccr2*$^{-/-}$ *Cx3cr1*$^{gfp/+}$ mice. Mean ± SEM are performed with four to six mice per group.

(B) Mean fluorescent intensities of CD115 expression on Ly6C$^-$ monocytes analyzed in (A). Mean ± SEM are performed with four to six mice each.

(C) Flow cytometric analysis of blood of *Cx3cr1*$^{creER/+}$*:R26-yfp* mice treated by tamoxifen gavage to induce excision of the STOP cassette from R26-YFP loci. Ly6C$^-$ blood monocytes were analyzed over a 2 week period for reporter gene expression. Mice were left untreated or treated with the CCR2 antibody MC21. Representative result of two experiments involving three mice per group.

(D) Mean fluorescent intensities of CD115 expression on Ly6C$^-$YFP$^+$ monocytes analyzed in (C). Mean ± SEM are performed with three mice per group.

(E) Mean fluorescent intensities of CD115 expression on Ly6C$^-$ monocytes of mice that received an injection of recombinant CSF-1. Mean ± SEM are performed with three mice each.

(F) Flow cytometric analysis of blood of tamoxifen-treated *Cx3cr1*$^{creER/+}$*:R26-yfp* mice. Ly6C$^-$ blood monocytes were analyzed over a 2 week period for reporter gene expression. Mice were left untreated, treated with the CCR2 antibody, or treated with a combination of MC21 and anti-CSF-1 R. Table summarizes half-lives of Ly6C$^-$ blood monocytes in the time window from 4 to 10 days, as determined by exponential trendline fitting.

See also Figures S5 and S6.

detected with a CD115 antibody (Figure 6B). One could postulate that in *Ccr2*$^{-/-}$ mice, Ly6C$^-$CX$_3$CR1^{hi} monocytes might compensate for the diminished replenishment from Ly6C$^+$ monocytes by extending their lifespan.

To directly investigate this possibility, we took advantage of our fate mapping system. A single 5 mg tamoxifen gavage of *Cx3cr1*^{creER}*:R26-yfp* mice induced in their CX$_3$CR1$^+$ cells the nuclear translocation of Cre recombinase and rearrangement of the YFP reporter locus, resulting in a discrete label of Ly6C$^-$ monocytes peaking by day 4 after gavage (Figure 6C). This label disappeared with time, and exponential trendline fitting from day 4 onward yielded a calculated steady-state half-life of Ly6C$^-$ blood monocytes of 2.2 days (r^2 = 0.996). If this half-life is critically determined by input from CCR2-expressing Ly6C$^+$ monocytes, then ablation of the latter via the anti-CCR2 regimen should affect it. Indeed, as seen in Figure 6C, MC21 treatment resulted in persistence of YFP-labeled Ly6C$^-$ monocytes in tamoxifen-treated *Cx3cr1*^{creER}*:R26-yfp* mice, extending their half-life from 2.2 days to 11 days (r^2 = 0.992). Once Ly6C$^+$ monocytes were restored, after cessation of MC21 treatment, Ly6C$^-$ cells regained their steady-state half-life and the population hence lost its YFP label. The depletion of CCR2-expressing Ly6C$^+$ monocytes was associated with a substantial downmodulation of detectable CD115 (CSF-1 receptor) on the Ly6C$^-$ monocytes, reminiscent of the situation in *Ccr2*$^{-/-}$ mice (Figure 6B), which was restored after MC21 withdrawal (Figure 6D).

Downmodulation of the CSF-1 receptor from the cell surface could be a reflection of engagement by its ligand and indeed administration of recombinant CSF-1 provided direct evidence that this can occur (Figure 6E). Because myeloid cells themselves control the circulating availability of CSF-1 (Bartocci et al., 1987; Tushinski et al., 1982), depletion of CSF-1R-expressing Ly6C$^+$ monocytes in *Ccr2*$^{-/-}$ mice or MC21-treated animals could potentially alter the availability of the factor. Although a blocking antibody against the receptor causes a substantial increase in circulating CSF-1 (MacDonald et al., 2010), we were not able to detect such an increase in the depleted animals (Figure S5). Nevertheless, to examine the function of CSF-1 in the extension of the circulation half-life of Ly6C$^-$ monocytes, we tested the impact of CSF-1 receptor

neutralization by using an antibody shown previously to ablate these cells in a time-dependent manner (MacDonald et al., 2010). Specifically, we coadministered tamoxifen-treated *Cx3cr1*^{creER}*:R26-yfp* mice with the MC21 regimen and CSF1R antibody. Interestingly and in support of a role of CSF-1 in the phenomenon, this protocol partially prevented the half-life extension of the YFP-labeled Ly6C$^-$ cells after depletion of Ly6C$^+$ monocytes (Figure 6F).

DISCUSSION

Here, we have reported a fate mapping analysis of the murine macrophage and monocyte compartment by taking advantage of mice that harbor genes encoding a GFP reporter or Cre recombinases in their CX_3CR1 loci. We have demonstrated that the major tissue macrophage populations in the liver, spleen, lung, and peritoneal cavity are established prenatally during mouse development and provided definitive support for the view that in the healthy adult organism, tissue macrophages can be maintained independent of monocyte input. In addition, we have provided evidence that CX_3CR1^{int}Ly6C$^+$ monocytes are, in steady state, an obligatory intermediate for the generation of CX_3CR1^{hi}Ly6C$^-$ monocytes and via their abundance control the circulation half-life of their progeny.

Recent studies have highlighted the existence of multiple pathways for the generation and maintenance of distinct mononuclear phagocyte subpopulations in the adult mouse (Geissmann et al., 2010). Accordingly, classical short-lived Flt3L-dependent DCs are continuously renewed by on-going hematopoiesis in the BM that feeds the periphery with dedicated DC precursors (Naik et al., 2007; Onai et al., 2007; Karsunky et al., 2003; Waskow et al., 2008). The temporal tissue residence profiles of macrophages have remained less well defined (Hume, 2006; Hume et al., 2002). Macrophages residing in the murine intestinal lamina propria are reported to have an estimated half-life of 3 weeks (Jaensson et al., 2008). Moreover, by using adoptive cell transfers, we and others provided evidence that these intestinal CX_3CR1^+ macrophages are continuously replenished from Ly6C$^+$ monocytes (Bogunovic et al., 2009; Varol et al., 2009). However, the gut mucosa-associated tissue probably represents an exception, because it is constitutively exposed to the luminal microflora and its products, which probably cause tonic low-grade inflammation. Other macrophage populations such as alveolar macrophages have been reported to persist for years (Murphy et al., 2008). Notably, however, previous studies on macrophage half-lives have been confounded by the fact that they involved irradiation regimens, which induce considerable damage to the tissue analyzed. Clear distinctions between inflammatory monocyte infiltrates and resident macrophages in these experimental setups might hence have been blurred. This complication was recently clarified in a series of studies that aimed to define the origins of brain microglia (Ginhoux et al., 2010).

Our comparative analysis of mice carrying a GFP reporter and conditional or constitutive active Cre recombinase genes in their *Cx3cr1* loci revealed that Kupffer cells, as well as lung, peritoneal, and splenic macrophages, are established before birth and remain in adulthood uncoupled from the steady-state monocyte pool. Notably, our results are well in line with the recent demonstration that epidermal Langerhans' cells are derived from primitive macrophages and fetal liver cells, but that in adulthood this compartment maintains itself independent from monocyte input (Hoeffel et al., 2012). Congruent with these data, patients with autosomal-dominant and sporadic monocytopenia present with an unaffected Langerhans' cell compartment (Bigley et al., 2011). What is more, our results are consistent with the recent evidence of the coexistence of two independent macrophage lineages in the mouse according to their differential dependence on the transcription factors PU.1 and c-myb (Schulz et al., 2012).

It is tempting to speculate that the reliance on resident macrophage populations established before birth represents an important design principle of tissue homeostasis ensuring robustness of the steady state. Accordingly, challenges such as injury and pathogen encounter will result in the recruitment of highly plastic monocytes, which critically but transiently (Ajami et al., 2011) contribute to inflammation and its resolution until restoration of homeostasis. Although our study focuses on the steady state, we have shown that an inflammatory stimulus can recruit monocytes whose descendants can seemingly integrate into the resident serosal, peritoneal cavity macrophage compartment. Future studies will have to address whether these cells become functionally equivalent to the resident cells and whether this applies also to solid tissues.

Monocytes can be divided into two main subpopulations, defined in the human as CD14^{hi}CD16$^{+/-}$ and CD14^{lo}CD16^{hi} cells and in the mouse as Ly6C$^+$$CX_3CR1^{int}$ and Ly6C$^-$$CX_3CR1^{hi}$ cells (Geissmann et al., 2003; Palframan et al., 2001). Earlier studies have provided circumstantial evidence that the monocyte subpopulations of the mouse are a differentiation series (Sunderkötter et al., 2004; Varol et al., 2007; Yrlid et al., 2006). Here we have reported the use of a series of complementary noninvasive approaches to interrogate the steady-state contribution of a Ly6C$^+$ monocyte intermediate to the generation of Ly6C$^-$ monocytes. First, we observed distinct temporal requirements for the creation of the two monocyte subsets with delayed appearance of the YFP reporter label in Ly6C$^-$ monocytes in *Cx3cr1*^{cre}*:R26-yfp* and *Cx3cr1*^{creER}*:R26-yfp* mice, as compared to *Cx3cr1*^{gfp} mice. Second, we have shown that a BrdU pulse label moves with time from Ly6C$^+$ monocytes to Ly6C$^-$ monocytes and ablation of the former cells abolishes appearance of the label in the latter. The data support earlier reports of an exceedingly short circulation half-life of Ly6C$^+$ monocytes (Liu et al., 2007; Varol et al., 2007), that at 0.8 days (19 hr), is longer than but comparable to that of short-lived neutrophils (11.4 hr) (Basu et al., 2002). Finally, we have shown by using competitive mixed chimeras that CCR2, which is critical for the BM exit of Ly6C$^+$ monocytes (Serbina and Pamer, 2006; Shi et al., 2011), is also required for the generation of Ly6C$^-$ monocytes. This strongly suggests that the majority, if not all, blood Ly6C$^-$ monocytes are derived from CCR2$^+$Ly6C$^+$ blood monocytes in the steady state (Figure S1).

We have also demonstrated that Ly6C$^-$ monocytes are generated independently in situ in the bone marrow. Thus, Ly6C$^-$ BM monocytes acquired the BrdU label well before the Ly6C$^-$ blood monocytes and appearance of the BrdU label was unaffected by the MC21-mediated ablation of Ly6C$^+$ blood monocytes. Moreover, also the differential dependence of Ly6C$^-$ BM and blood

monocytes on CCR2 expression, as well as the resilience of BrdU incorporation of the latter to the MC21 regimen, suggest that these cells are generated in BM and blood, respectively. It remains to be shown whether Ly6C$^-$ BM monocytes arise like Ly6C$^-$ blood monocytes via a Ly6C$^+$ intermediate or can be generated directly from MDPs, because the MC21 treatment spares Ly6C$^+$ BM monocytes. The function of Ly6C$^-$ blood monocytes has been proposed to be to patrol and survey endothelial integrity (Auffray et al., 2007). When cultured in vitro with CSF-1 (M-CSF) or CSF-2 (GM-CSF), both Ly6C$^+$ and Ly6C$^-$ monocytes can differentiate into macrophages and DCs, respectively (data not shown). However, whereas Ly6C$^+$ monocytes are established in vivo precursors of intestinal macrophages, Ly6C$^-$ monocytes fail to do so (Varol et al., 2009). Moreover, reports demonstrating an in vivo potential of these cells to act as resident mononuclear phagocyte precursors are scarce (Landsman and Jung, 2007; Nahrendorf et al., 2007). Paradoxically, in humans the supposed functional equivalent, the CD14$^-$CD16$^+$ nonclassical monocytes, is normally much less abundant but increases in response to chronic inflammatory stimuli (Ziegler-Heitbrock et al., 2010). In light of our present findings, we propose that Ly6C$^-$ blood monocytes represent the homeostatic default product of short-lived Ly6C$^+$ monocytes in the blood that surveys endothelial integrity (Auffray et al., 2007), as terminally differentiated blood-resident macrophages.

Our study has revealed that the lifespan of Ly6C$^-$ blood monocytes is affected by the prevalence of Ly6C$^+$ blood monocytes, their direct precursors. Thus, whereas these cells display in unmanipulated steady state a half-life of 2 days, their half-life is extended to 11 days when the Ly6C$^+$ precursors are ablated. Prolonged neutralization of the CSF-1 receptor has been shown to result in the selective loss of Ly6C$^-$ blood monocytes (MacDonald et al., 2010). Conversely, ablation of CD115$^+$Ly6C$^+$ blood monocytes could result in an increase of plasma CSF-1 promoting survival of Ly6C$^-$ blood monocytes. Interestingly, CD115 amounts were found downmodulated on Ly6C$^-$ blood monocytes of *CCR2*$^{-/-}$ mice. Moreover, expression of CD115 was also reduced in mice in which Ly6C$^+$ blood monocytes were ablated, but CD115 was rapidly restored upon withdrawal of the MC21 treatment. Extension of the half-life in response to the MC21 treatment was partially impaired by concomitant neutralization of the CSF-1 receptor, suggesting that limited CSF-1 availability in the serum is associated with this phenomenon and that Ly6C$^+$ monocytes constitute a sink for this critical survival factor. Interestingly, CSF-1 has been reported to induce the NOR1 (Pei et al., 2005), an orphan nuclear receptor and member of the steroid thyroid receptor family, which also comprises Nur77 (Nr4A) that was recently implicated in the control of Ly6C$^-$ monocyte survival (Hanna et al., 2011).

Although the exact molecular pathway underlying the regulation remains to be elucidated, our study establishes that Ly6C$^+$ monocytes are not only obligatory steady-state precursors of Ly6C$^-$ monocytes, but also restrict the lifespan of their otherwise long-lived progeny. Conversely, the unique ability of Ly6C$^-$ monocytes to extend their circulation half-life could be a mechanism to ensure robust maintenance of a critical Ly6C$^-$ population size, potentially required for efficient surveillance of endothelial integrity (Auffray et al., 2007), even under conditions when Ly6C$^+$ monocytes are withdrawn from their precursor task to sites of inflammation.

Collectively, we have provided here a fate mapping study of murine mononuclear phagocytes, focusing on macrophages and monocytes. Our results highlight the coexistence of distinct tissue-resident macrophage populations in the mammalian organism, including cellular compartments that are established during development from primitive macrophages or fetal liver cells but remain during health independent from monocyte input. Within the monocyte compartment, we have established Ly6C$^+$ blood monocytes as obligatory precursor cells of Ly6C$^-$ blood monocytes and reveal that the lifespan of these cells can be dynamically regulated to maintain a stable Ly6C$^-$ blood monocyte compartment in case of Ly6C$^+$ blood monocyte shortage. Full appreciation of the monocyte and macrophage complexity in the mammalian organism will require future studies exploring the existence of distinctive molecular and functional characteristics of these distinct populations that might pave the way for rational strategies to manipulate mononuclear phagocytes for therapeutic purposes.

EXPERIMENTAL PROCEDURES

Mice

The following 6- to 10-week-old mouse strains were used: *Cx3cr1*$^{gfp/+}$ mice (Jung et al., 2000), *Pgk-cre* mice (Lallemand et al., 1998), *Ccr2*$^{-/-}$ mice (Boring et al., 1997), and *Ccr2*$^{-/-}$:*Cx3cr1*$^{gfp/+}$ mice. To analyze recombinase activity of *Cx3cr1*^{cre} or *Cx3cr1*^{creER} mice (for generation information, see Supplemental Experimental Procedures), these animals were crossed to *Rosa-26-yfp* (Srinivas et al., 2001) or *Rosa-26-rfp* (Luche et al., 2007) reporter mice. BM chimera animals were lethally irradiated and reconstituted with donor BM (see Supplemental Experimental Procedures for further details). All mice studied were on C57BL/6 background, maintained under specific-pathogen-free conditions, and handled under protocols approved by the Weizmann Institute Animal Care Committee (IACUC) in accordance with international guidelines.

Tamoxifen Treatment

Tamoxifen was administered either orally or subcutaneously. See Supplemental Experimental Procedures for further details.

Isolation of Tissue Samples

Blood, BM, and tissue samples were prepared as described previously (Yona et al., 2010). Single-cell suspensions were then stained and subsequently analyzed by polychromatic flow cytometry. A detailed description of tissue mononuclear phagocyte isolation and antibody clones used throughout this study can be found in the Supplemental Experimental Procedures.

Isolation of Monocytes for Adoptive Transfers

BM cells were harvested and mononuclear cells enriched by density gradient. Splenic monocytes were isolated by MACS enrichment with biotinylated CD115 antibody and streptavidin-coupled magnetic beads (Miltenyi Biotec). Ly6C^{hi} monocytes were identified as CD11b$^+$CD115$^+$ and Gr-1$^+$, cells were purified by high-speed cell sorting and with a FACS Aria (Becton-Dickson). Sorted cells were resuspended and injected i.v. into congenic CD45.1 WT mice. See Supplemental Experimental Procedures for further details.

BrdU Pulsing

Mice were treated with three injections of 2 mg BrdU (5-bromo-2-deoxyuridine; BD PharMingen) i.p. 3 hr apart or a single 2 mg i.p injection. To assess BrdU incorporation, monocytes were stained, fixed, and permeabilized according to the manufacturer's instructions prior to analysis by flow cytometry. See Supplemental Experimental Procedures for further details.

Antibody and Cytokine Treatment

Mice were treated with 150 μl of CCR2 mAb (clone: MC21) conditioned media i.p. To block CD115, mice were treated with CD115 mAb (clone: M279, MacDonald et al., 2010) i.p. Recombinant M-CSF (PeproTech) (25 μg/mouse) was given i.p. See Supplemental Experimental Procedures for further details.

Histology

Mice were anesthetized and perfused with 30 ml of PBS and organs were excised and fixed in paraformaldehyde prior to being imbedded in OCT. Cryostatic sections were post-fixed and stained. Analysis by confocal laser scanning microscopy was performed with a Zeiss LSM510 microscope. Image acquisition was processed with Zeiss LSM Image browser software. See Supplemental Experimental Procedures for further details.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and six figures and can be found with this article online at http://dx.doi.org/10.1016/j.immuni.2012.12.001.

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