Fate Mapping via Ms4a3-Expression History Traces Monocyte-Derived Cells

Graphical Abstract

Highlights

- Ms4a3 is specifically and transiently expressed by GMPs in the bone marrow
- MDPs do not arise from GMPs and do not give rise to cMoPs
- Ms4a3-based models specifically and efficiently fate map monocytes and granulocytes
- Distinguish monocyte- versus embryonic-derived RTMs in steady state and inflammation

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In Brief

A fate-mapping model provides insights into the ontogeny of specific monocyte populations and their contributions to the tissue-resident macrophage pools during homeostasis and inflammation.
Fate Mapping via Ms4a3-Expression History Traces Monocyte-Derived Cells

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SUMMARY

Most tissue-resident macrophage (RTM) populations are seeded by waves of embryonic hematopoiesis and are self-maintained independently of a bone marrow contribution during adulthood. A proportion of RTMs, however, is constantly replaced by blood monocytes, and their functions compared to embryonic RTMs remain unclear. The kinetics and extent of the contribution of circulating monocytes to RTM replacement during homeostasis, inflammation, and disease are highly debated. Here, we identified Ms4a3 as a specific gene expressed by granulocyte-monocyte progenitors (GMPs) and subsequently generated Ms4a3<sup>Cre</sup> reporter, Ms4a3<sup>Cre</sup>, and Ms4a3<sup>CreERT2</sup> fate-mapping models. These models traced efficiently monocytes and granulocytes, but no lymphocytes or tissue dendritic cells. Using these models, we precisely quantified the contribution of monocytes to the RTM pool during homeostasis and inflammation. The unambiguous identification of monocyte-derived cells will permit future studies of their function under any condition.

INTRODUCTION

Tissue-resident macrophages (RTMs) have vital roles in tissue homeostasis, inflammation, and remodeling (Ginhoux and Jung, 2014), but their origins and maintenance are debated. Macrophages were originally proposed to be derived from circulating monocytes (van Furth and Cohn, 1968), but recent studies have challenged this model, causing an important conceptual frameshift in the field (Ginhoux et al., 2010; Guilliams et al., 2013; Hashimoto et al., 2013; Hoeffel et al., 2012; Jenkins et al., 2011; Schulz et al., 2012; Yona et al., 2013) (reviewed in Ginhoux and Guilliams, 2016). Namely, these studies revealed that adult RTMs arise from successive waves of embryonic and adult hematopoiesis (reviewed in Hoeffel and Ginhoux, 2015), and the extent of contribution and the kinetics of these waves to each RTM population is tissue specific (Ginhoux and Guilliams, 2016).

The exact contribution of adult definitive hematopoiesis to RTMs is an area of intense investigation. Using parabiosis and genetic fate-mapping approaches, Hashimoto et al. showed that some RTMs (such as lung alveolar macrophages [AMs], red pulp macrophages, and peritoneal macrophages) self-maintain locally throughout adult life with minimal contribution from circulating monocytes (Hashimoto et al., 2013). Others have shown that barrier tissues, such as the gut and dermis, have a notable monocyte contribution (Bain et al., 2014; Tamoutounour et al., 2013). Furthermore, bone marrow (BM)-derived monocytes can differentiate into arterial macrophages immediately after birth and locally self-renew from this point (Ensarn et al., 2016), suggesting that this tissue is only temporarily “open” at birth but remains “closed” during adulthood. Adult tissues can thus be classified as (1) closed, with no steady-state monocyte recruitment (brain, epidermis, lung, and liver) and self-maintained throughout life, without or with only minimal contribution of blood monocytes; (2) open, with fast steady-state recruitment (gut and dermis); or (3) open, with slow steady-state recruitment (heart and pancreas) (Ginhoux and Guilliams, 2016).

The mechanisms behind these differential renewal patterns are not fully understood and may be controlled by the tissue-specific microenvironment, sex, and/or other factors. Bain et al. showed that peritoneal macrophage renewal follows a sexually dimorphic pattern, with more monocytes contributing to peritoneal macrophages in males than in females (Bain et al., 2016). The situation during inflammation is even more complicated, as partial RTM depletion occurs alongside inflammatory cell recruitment, including neutrophils and monocytes (Guilliams and Scott, 2017). These monocytes may potentially contribute to RTMs upon resolution of inflammation (Guilliams and Scott, 2017). During *Listeria monocytogenes* infection, liver-resident macrophages undergo necroptosis in conjunction with rapid monocyte infiltration to give rise to monocyte-derived Kupffer cells (Blériot et al., 2015). In an opposite scenario,
Figure 1. Ms4a3 Is Specifically Expressed by Monocyte-Committed Progenitors

(A) CMap analysis of individual single-cell populations showing enrichment for monocyte or DC signature genes. Cells with a positive CMap score were denoted as monocyte-primed cells; cells with a negative CMap score were denoted as DC-primed cells. Cells in the red rectangle (monocyte-committed cells) and blue rectangle (DC-committed cells) were used for DEG analysis.

(B) Heatmap of the top 50 DEGs upregulated in monocyte-primed cells (indicated by the red rectangle in A) than in DC-primed cells (indicated by the blue rectangle in A). Yellow, high expression; purple, low expression.

(C) Violin plot of Ms4a3, Cx3cr1, and Lyz2 expression in indicated populations. y axis represents the TPM (transcript per million reads) of the gene in each single cell.

(legend continued on next page)
during type 2 inflammation induced by *Litomosoides sigmodontis* infection, it is local macrophage proliferation rather than inflammatory cell recruitment that controls macrophage expansion in C57BL/6 mice (Jenkins et al., 2011). These observations suggest that the developmental stage, and tissue-specific and inflammation-specific conditions, controls the origins of RTMs under steady state and during inflammation. There is thus a need to precisely identify the origins of RTMs under any condition.

Monocyte fate-mapping models, including Cx3cr1Cre or Cx3cr1CreERT2 (Yona et al., 2013) and LyzMCre (Clausen et al., 1999) are not fully accurate, labeling either dendritic cells (DCs) or RTMs respectively. Here, we aimed to develop a new fate-mapping mouse model specific for monocyte progenitors that could precisely measure the contribution of monocytes to RTMs under any condition and discern monocytes from DCs. The earliest monopotent BM progenitors giving rise to monocytes are common monocyte progenitors (cMoPs) (Hettinger et al., 2008), or RTMs respectively. Here, we aimed to develop a new fate-mapper, and thus performed single-cell profiling of progenitor cells and subfamily A, member 3 (MHCII expression in the indicated cell types in the peripheral blood and spleen (n = 4–6). Error bars, SEM.

We performed a single-cell transcriptomic analysis of monocyte and DC progenitors by scRNA-seq using the C1 Fluidigm platform (Figure S1A for workflow). We sorted BM cMoPs (Lin−CD117−CD115−CD135+Ly6C”), BM Ly6C+ monocytes (Lin−CD117+CD115−CD135+Ly6C”), and blood Ly6C+ monocytes (CD115+CD11b−Ly6C”) from wild-type (WT) C57BL/6 mice by fluorescence-activated cell sorting (FACS) (Figures S1B and S1C for gating strategy) and generated transcriptional profiles for each individual cell (n = 38 for blood Ly6C+ monocytes, n = 66 for BM cMoPs, n = 57 for BM Ly6C− monocytes). We referred to our previously published dataset for MDPs (Lin−CD11c−MHCII−CD135−CD115−CD117−), CDPs (Lin+CD11c−MHCII−CD135−CD115−CD117−), and pre-DCs (Lin+CD11c+MHCII−CD135−CD172a−) (Schlitzer et al., 2015). To identify putative monocyte-primed versus DC lineage-primed cells within these precursors, we compared the transcriptomic signatures of each single cell to DC versus monocyte- and/or macrophage-specific signatures (Schlitzer et al., 2015) using Connectivity Map (CMap) analysis (Lamb et al., 2006) (Figure 1A; see STAR Methods for bioinformatics analysis details). Here, we identified within the whole MDP stage single MDPs committed to the monocytic lineage versus the DC lineage (Figure 1A).

As expected, progenitor populations downstream of the MDP stage mostly exhibited either monocyte (cMoPs and monocytes) or DC (CDPs and pre-DCs) commitment. We identified the differentially expressed genes (DEGs) (see STAR Methods for bioinformatics analysis details) between monocyte-primed progenitors (red rectangle in Figure 1A) versus DC-primed progenitors (blue rectangle in Figure 1A), and selected the top 50 up-regulated genes in monocyte-primed progenitors compared to DC-primed progenitors (Figure 1B). After screening the expression profiles of these 50 genes in our scRNA-seq data and the ImmGen (Heng et al., 2008) and bioGPS databases (Wu et al., 2009), we identified Ms4a3 as a potential candidate gene because of its high and specific expression profile in BM monocyte progenitors (Figures 1B, 1C, and S1D). Ms4a3 is a member of the membrane-spanning 4A gene family and is closely related to CD20 and the beta-subunit of the high-affinity immunoglobulin E (IgE) receptor (FcrRIß) (Hulett et al., 2001). Using the ImmGen database, we found that Ms4a3 is highly expressed in BM GMPs, lowly expressed in MDPs (Figure S1E), and not expressed in DCs or various RTMs (Figure S1F). Exploring the bioGPS database, we found Ms4a3 to be only expressed in the BM, predominantly by GMPs (Figure S1G). With our single-cell sequencing data, we visualized Ms4a3 expression overlaid on

**RESULTS**

**Ms4a3 Is Specifically Expressed by Monocyte-Committed Progenitors**

We first aimed to identify a suitable gene to generate a Cre-recombinase-based monocyte fate-mapping model, by profiling the genes expressed in BM cMoPs and monocytes, but not in DC progenitors, such as common dendritic cell progenitors (CDPs), circulating DC precursors (pre-DCs) (to distinguish monocytes versus DCs), or differentiated macrophages (to distinguish monocyte contribution versus intrinsic expression in macrophages). MDPs have bi-potential, giving rise to both DCs and monocytes (Auffray et al., 2009). We hypothesized that within the MDP population, MDPs that are committed to the monocyte lineage co-exist with MDPs committed to the DC lineage. We thus aimed to find a gene that is uniquely expressed in MDPs that are committed to the monocyte lineage.

We performed a single-cell transcriptomic analysis of monocyte and DC progenitors by scRNA-seq using the C1 Fluidigm platform (Figure S1A for workflow). We sorted BM cMoPs (Lin−CD117−CD115−CD135+Ly6C”), BM Ly6C+ monocytes (Lin−CD117+CD115−CD135+Ly6C”), and blood Ly6C+ monocytes (CD115+CD11b−Ly6C”) from wild-type (WT) C57BL/6 mice by fluorescence-activated cell sorting (FACS) (Figures S1B and S1C for gating strategy) and generated transcriptional profiles for each individual cell (n = 38 for blood Ly6C+ monocytes, n = 66 for BM cMoPs, n = 57 for BM Ly6C− monocytes). We referred to our previously published dataset for MDPs (Lin−CD11c−MHCII−CD135−CD115−CD117−), CDPs (Lin+CD11c−MHCII−CD135−CD115−CD117−), and pre-DCs (Lin+CD11c+MHCII−CD135−CD172a−) (Schlitzer et al., 2015). To identify putative monocyte-primed versus DC lineage-primed cells within these precursors, we compared the transcriptomic signatures of each single cell to DC versus monocyte- and/or macrophage-specific signatures (Schlitzer et al., 2015) using Connectivity Map (CMap) analysis (Lamb et al., 2006) (Figure 1A; see STAR Methods for bioinformatics analysis details). Here, we identified within the whole MDP stage single MDPs committed to the monocytic lineage versus the DC lineage (Figure 1A).

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the $t$-distributed stochastic neighbor embedding (tSNE) plot (Figure 1D). In this analysis, Ms4a3 was highly expressed in cMoPs but not in CDPs, suggesting its potential utility to distinguish monocytes from DCs in a fate-mapping approach (Figures 1C and 1D). We also compared the expression of Ms4a3 with Cx3cr1 and Lyz2, genes used to previously make monocyte fate-mapping models. In contrast to Ms4a3, which was only expressed in cMoPs but not in terminally differentiated blood Ly6C$^+$ monocytes, Lyz2 was expressed at a higher level in terminally differentiated Ly6C$^+$ monocytes than in cMoPs and was also expressed by pre-DCs, while Cx3cr1 was expressed by both monocyte-primed cells and DC-primed cells (Figures 1C and 1D). We also verified at the single-cell level that Ms4a3 was expressed by GMPs (single-cell dataset published in Dress et al. [2019]) (Figure 1E). To confirm these data, we sorted BM CMPs, GMPs, granulocyte progenitors (GPs), cMoPs, MDPs, CDPs, and Ly6C$^+$ monocytes (Figure S2A for gating strategy) and profiled Ms4a3 expression by quantitative real-time polymerase chain reaction (qRT-PCR). Consistent to the scRNA-seq data and the expression data from the ImmGen and bioGPS databases, we detected high Ms4a3 expression in BM GMPs and cMoPs (Figure 1F). In summary, our single-cell sequencing data, qRT-PCR data, and expression data from public databases suggest that Ms4a3 is highly and specifically expressed in GMPs, GPs, and cMoPs and might be more suitable to specifically fate map monocytes than Cx3cr1 and Lyz2.

**Ms4a3 Is Not Expressed by RTMs or DCs**

Here, we hypothesized that Ms4a3 is a suitable target gene for building a monocyte fate mapper that could precisely measure the contribution of monocytes to RTMs on the condition that it is not expressed in mature RTMs or DCs. We first verified that Ms4a3 was not expressed by RTMs or DCs in the ImmGen and bioGPS databases (Figures S1F and S1G). To confirm these data, we sorted cell populations of interest from various tissues and profiled Ms4a3 expression by qRT-PCR (Figures S2B–S2D for gating strategy). Ms4a3 was expressed in basophils and neutrophils in the blood but not expressed in lymphocytes (T cells, B cells, and natural killer [NK] cells), mature monocytes, eosinophils, or splenic DCs (conventional dendritic cell 1 [cDC1], cDC2, and plasmacytoid DCs [pDCs]) (Figure 1G). We next measured Ms4a3 expression in several RTM populations and detected no notable expression in peritoneal macrophages, lung AMs, liver KCs, gut macrophages, microglia, or Langerhans cells (LCs) (Figures 1H and S2E–S2J for gating strategy).

Ms4a3 is a membrane-associated protein located in the perinuclear area but not at the cell surface (Donato et al., 2002), and no antibodies for mouse Ms4a3 are commercially available, limiting expression detection by flow cytometry. To monitor the expression of Ms4a3 at the single-cell level, we developed a Ms4a3 reporter mouse by inserting an Ires-Cre cassette downstream of the Ms4a3 stop codon (denoted as Ms4a3$^{Cre^-Rosa}$ mouse hereafter) (Figure S3A). In this model, we expected tdTomato to be expressed as a faithful reporter in Ms4a3-expressing cells. Indeed, in the BM of Ms4a3$^{Cre^-Rosa}$ mice, the earliest tdTomato signal appeared in GMPs and was detectable in cMoPs and monocytes (albeit to a lesser extent) (Figure 2A), which is in agreement with our qRT-PCR profiling (Figure 1F).

Furthermore, we confirmed high tdTomato protein expression levels in peripheral blood basophils and neutrophils and a low level in monocytes, likely reflecting the residual expression of the tdTomato protein initiated in the BM and no expression in lymphoid lineages (Figures 2B and 2C), splenic cDC1, cDC2, or pDCs (Figure 2D). Profiling of tdTomato mRNA by qRT-PCR led to a similar profile with high mRNA levels in BM GMPs and cMoPs but very low levels in blood monocytes (Figure 2E). We compared these results to the commonly used monocyte reporter model Cx3cr1-GFP mice (Jung et al., 2000), BM cMoPs and monocytes expressed the GFP reporter, but also MDPs and CDPs (Figure S3B), consistent with a previous report (Yona et al., 2013). In the blood, monocytes expressed a high level of Cx3cr1-GFP, besides basophils and some NK cells (Figures S3C and S3D). In the spleen, some DCs were positive for Cx3cr1-GFP (Figure S3E) while negative for Ms4a3-tdTomato in the Ms4a3$^{Cre^-Rosa}$ model (Figure 2D). In a Ms4a3$^{Cre^-Rosa}$, Cx3cr1-GFP model, the overlap between tdTomato and GFP could only be detected in monocytes and some basophils in the peripheral blood (Figure 2F). From Ly6C$^+$ to Ly6C$^-$ monocytes, the expression of GFP increased while the tdTomato expression decreased (Figure 2G). These populations can be clearly identified based on the differential expression of Cx3cr1-GFP and Ms4a3-tdTomato (Figure S3F). These data support the concept that Ms4a3-based fate-mapping models would help to distinguish monocytes from the cells of the DC lineage.

We next analyzed tdTomato expression in several RTM populations (brain microglia, skin LCs, liver KCs, lung AMs, splenic macrophages, peritoneal macrophages, kidney macrophages, gut macrophages, and dermal macrophages) in young (8-week-old) and old (6-month-old) Ms4a3$^{Cre^-Rosa}$ mice, and consistent with our qRT-PCR data (Figure 1G), we found that none of them expressed tdTomato (Figures S4A–S4I). We also analyzed the expression of the GFP reporter in RTMs of Cx3cr1-GFP mice, and consistent with a previous report (Yona et al., 2013), microglia, gut macrophages, and kidney macrophages express in steady state a high level of the reporter (Figures S4A–S4I), which limits the use of this model to distinguish embryonic macrophages from monocyte-derived macrophages. Collectively, these data suggest that Ms4a3 is a specific marker for BM GMP and cMoP stages and may be suitable for labeling GMPs and their progenies, including monocytes.

**Ms4a3$^{Cre^-Rosa}$ Model Specifically and Efficiently Fate Maps Granulocytes and Monocytes**

We next generated a Ms4a3$^{Cre^-}$ fate-mapper mouse model by inserting an Ires-Cre cassette downstream of the Ms4a3 stop codon (Figure S5A) and crossed this strain with the Rosa$^{26Gtato}$ reporter strain. In the resulting Ms4a3$^{Cre^-Rosa}$ model, Cre recombinase will delete the Stop signal adjacent to tdTomato in Ms4a3-expressing cells, resulting in irreversible and persistent tdTomato red fluorescent protein expression in Ms4a3-expressing cells and their progeny (Figure S5A). According to the BioGPS database, where Ms4a3 is only expressed in the BM (Figure S1G), we predicted no tdTomato labeling in Ms4a3$^{Cre^-Rosa}$ non-hematopoietic cells. Indeed, we found no tdTomato labeling in non-hematopoietic CD45$^-$ cells in the brain, skin, liver, lung, kidney, pancreas, heart, salivary gland, colon, or small intestine. We did, however, observe ~60%
Figure 2. tdTomato Expression in Ms4a3\textsuperscript{Tr/T} Mice
(A) tdTomato expression in indicated progenitor cell types in the BM of Ms4a3\textsuperscript{Tr/T} (filled gray) and WT (open) mice.
(B) tdTomato expression in indicated cell types in the peripheral blood of Ms4a3\textsuperscript{Tr/T} mice (filled gray) and WT (open) mice.
(C) tSNE plot shows the intensity of tdTomato in peripheral blood cells. The color indicates the expression intensity of tdTomato: red, high expression; blue, low expression.
(D) tdTomato expression in splenic cDCs and pDCs in Ms4a3\textsuperscript{Tr/T} mice (filled gray) and WT (open) mice. Each experiment was repeated at least 3 times with 2–3 replicates, and a representative plot is shown.
(E) qRT-PCR analysis of Ms4a3 and tdTomato expression in indicated populations (n = 4). Expression was normalized to Gapdh (2\textsuperscript{-deltaCt}). Error bars, SEM.
(F) tSNE plot shows the expression of Cx3cr1-GFP and Ms4a3-tdTomato in the peripheral blood of Cx3cr1\textsuperscript{Cre}\textsuperscript{+};Ms4a3\textsuperscript{Tr/T} mice. Red, high expression; blue, low expression.
(G) Flow plots show the expression of Cx3cr1-GFP and Ms4a3-tdTomato in monocytes, waterfall from Ly6C\textsuperscript{hi} to Ly6C\textsuperscript{lo}.
See also Figures S3 and S4.
tdTomato labeling in CD45$^+$ cells in the testicles (Figure S5B) and confirmed low tdTomato expression in sperm by microscopy (Figure S5C), we did not detect any tdTomato expression in adult testicles in Ms4a3CreERT2 reporter mice (Figure S5D). Going forward, to circumvent potential biases generated by germ-line recombination, we did not use Ms4a3CreERT2-RosaTdt male mice as breeders.

We then analyzed tdTomato labeling in leukocyte lineages in the peripheral blood, spleen, and various tissues of our Ms4a3CreERT2-RosaTdt model. As expected, lymphoid cells in the peripheral blood, including T cells (0.03% ± 0.001%), B cells (0.004% ± 0.001%), and NK cells (0.120% ± 0.027%), were not labeled, while neutrophils (99.9% ± 0.048%), basophils (94.1% ± 1.151%), eosinophils (99.5% ± 0.309%), Ly6C$^+$ monocytes (97.3% ± 0.320%) and Ly6C$^+$ monocytes (95.1% ± 0.530%) were highly labeled (Figures 3A and S5E). When performing a reverse analysis by first gating on tdTomato$^+$ cells, almost all tdTomato$^+$ cells were CD172a$^+$CD11b$^+$ myeloid cells (Figure 3B). tSNE analysis clearly showed that tdTomato$^+$ cells were neutrophils, basophils, eosinophils, and monocytes (Figure 3C).

We then analyzed tdTomato labeling in splenic DCs. Here, we detected a low level of labeling in XCR1$^+$ cDC1s (3.585% ± 0.652%) and CD172a$^+$ cDC2s (6.000% ± 1.012%) and no labeling in MHCI$^+$B220$^+$CD172a$^+$ pDCs (0.075% ± 0.033%) (Figures 3D–3F). We confirmed these findings by microscopy, where we observed negligible overlap between tdTomato$^+$ (red) and CD11c$^+$ (cyan) cells (considered DCs) in the spleen (Figures 3G and 3H). Finally, we did a parallel comparison of Ms4a3CreERT2-RosaTdt with Cx3cr1CreERT2-RosaTdt. The latter mainly labels monocytes and DCs, but also with very high background labeling in lymphocytes and granulocytes (Figures S5F and S5G), while Ms4a3CreERT2-RosaTdt only specifically labels monocytes and granulocytes. Taken together, the Ms4a3CreERT2-RosaTdt mouse model permits faithful genetic marking of monocytes and granulocytes, but not DCs or lymphocytes.

**Ms4a3CreERT2-RosaTdt Model Specifically and Efficiently Fate Maps Granulocytes and Monocytes**

We also developed a tamoxifen-inducible model (named Ms4a3CreERT2) by inserting an Ires-CreERT2 cassette downstream of the Ms4a3 stop codon and crossed this strain with the Rosa26tdTomato reporter strain (Figure S5H). This conditional fate-mapping model contains a Cre recombinase fused to a mutant estrogen ligand–binding domain (CreERT2) that requires the estrogen antagonist tamoxifen for activity (Feil et al., 1997). Upon tamoxifen injection, Ms4a3-expressing cells will start to express the tdTomato reporter in an irreversible fashion, allowing us to fate map the contribution of GMPs in a time-controlled manner.

Ms4a3CreERT2-RosaTdt mice were given tamoxifen for 4 successive days by intraperitoneal (i.p.) injection and analyzed on day 7 (Figure S5I). Consistent with Ms4a3CreERT2-RosaTdt mice, neutrophils (99.6% ± 0.05%), eosinophils (91.8% ± 0.87%), basophils (98.3% ± 0.34%), and Ly6C$^+$ monocytes (94.0% ± 0.78%) were labeled (Figures 3I and 3J). However, Ly6C$^+$ monocytes were labeled with a lower percentage (27.9% ± 3.33%) (Figure 3J) and in a more delayed fashion than Ly6C$^+$ monocytes (Figure 3K), in agreement with the developmental relationship between these subsets, with Ly6C$^+$ monocytes giving rise to Ly6C$^+$ monocytes (Yona et al., 2013). A similar level of labeling was observed in Ly6C$^+$ monocytes from the Cx3cr1CreERT2 mice (91.4% ± 2.38%) (Figures S5J and S5K). In contrast, DCs (62.7% ± 1.01% for cDC1, 37.4% ± 1.47% for cDC2, and 67.0% ± 2.29% for pDC) were labeled in Cx3cr1CreERT2-RosaTdt mice (Figure S5K) while not in Ms4a3CreERT2-RosaTdt mice (2.20% ± 0.40% for cDC1, 4.35% ± 0.60% for cDC2, and 0.10% ± 0.04% for pDC) (Figure S5J). In summary, the Ms4a3CreERT2-RosaTdt model permits the faithful genetic marking of monocytes and granulocytes but not DCs or lymphocytes.

**Ms4a3 Labels GMPs but Not MDPs in the BM**

Monocytes are proposed to arise from the hierarchical model of CMP → GMP → MDP → cMoP → monocyte (Ginhoux and Jung, 2014; Guilliams et al., 2018; Terry and Miller, 2014). However, this model has been recently challenged by Yanez et al., who proposed that MDPs arise directly from CMPs independently of GMPs and that GMPs and MDPs give rise to distinct monocytes via similar pathways through monocyte-committed progenitors (MP) and cMoPs, respectively (Yanez et al., 2017). To test this hypothesis and to explore at which stage monocytes...
and granulocytes were labeled; we analyzed tdTomato labeling in BM progenitor cells in Ms4a3\(^{Cre}\)–Rosa\(^{TdT}\) mice. As predicted, GMPs were the earliest progenitors to be highly labeled (68.7\% ± 1.58\%), and almost all GMP progenies, such as cMoPs (93.5\% ± 0.251\%) and GPs (91.8\% ± 0.490\%) were labeled (Figures 4A and 4B). However, MDPs were lowly labeled (4.5\% ± 0.23\%) (Figures 4A and 4B). Reverse analysis by first gating on tdTomato\(^{+}\) cells showed that almost all tdTomato\(^{+}\) cells were CD16/32\(^{hi}\), containing GMPs, cMoPs, GPs, monocytes, and neutrophils (Figures 4C and S6A), while tdTomato\(^{-}\) cells were mainly CD16/32\(^{lo}\), containing CMPs, MDPs, and DC precursors (Figures 4C and S6B).

Importantly, MDPs were initially described as Cx3cr1-GFP\(^{+}\) CD117\(^{+}\)CD11b\(^{-}\) cells (Fogg et al., 2006), but alternate gating strategies to identify them were proposed, bypassing the need of the Cx3cr1\(^{Gfp}\) reporter model (Hettinger et al., 2013; Yanez et al., 2017). Thus, to be able to identify MDPs as initially described, we crossed our Ms4a3\(^{Cre}\)–Rosa\(^{TdT}\) mice with Cx3cr1\(^{Gfp}\) mice. In the initial gating strategy used by Fogg et al., we found part of the MDPs (Lin\(^{-}\)Cx3cr1-GFP\(^{+}\)CD117\(^{+}\)CD11b\(^{-}\)) to be tdTomato\(^{+}\) (Figure S6C). However, this fraction has a similar phenotype to cMoPs (Lin\(^{-}\)CD115\(^{+}\)CD117\(^{+}\)CD135\(^{-}\)Ly6C\(^{+}\)) (Figure S6C) described by Hettinger et al. (2013), suggesting that most of the Cx3cr1-GFP\(^{+}\)tdTomato\(^{+}\) fraction in the initial Fogg et al. MDP gating are likely corresponding to cMoPs. We also applied the gating strategies from Yanez et al. (2017) (Figures 4D and S6D) and Hettinger et al. (2013) (Figures 4E and S6E), in which the Cx3cr1-GFP reporter was not used to identify MDPs. In both gating strategies, the Ms4a3\(^{Cre}\)–Rosa\(^{TdT}\) model labeled cMoPs and monocytes but...
did not label MDPs (Figures 4D and 4E). These data support the notion that MDPs do not arise from GMPs but arise from CMPs (Yanez et al., 2017).

**MDPs Do Not Arise from GMPs and Do Not Give Rise to cMoPs**

Our Ms4a3Cre-RosaTdt model labeled GMPs, cMoPs, and monocytes but did not label CMPs and MDPs, in agreement with the notion that MDPs do not arise from GMPs but arise from CMPs (Yanez et al., 2017). However, not all GMPs were labeled (68.7% ± 1.58%), and it could be hypothesized that unlabeled GMPs could give rise to MDPs. To test whether MDPs could still arise from the tdTomato positivity fraction of GMPs, we sorted tdTomato−GMPs and tdTomato−MDPs and co-cultured them with CD45.1 BM cells in the presence of 50 ng/mL murine M-CSF (CSF-1) for 2 days. Importantly, we did not observe MDP (CD11C+CD16/32−CD135−CD115+ or DC (CD11c+MHCIIhi) production by tdTomato−GMPs (Figure 5A) but rather the generation of tdTomato+ cMoPs (CD11B+CD16/32−CD135−CD115+Ly6C+) (Figure 5A), confirming that MDPs do not arise from GMPs. However, our MDP and cMoP labeling findings (cMoPs were highly labeled in the Ms4a3Cre-RosaTdt model, while MDPs were not) as well as the generation of tdTomato+ cMoPs by GMPs but not by MDPs in vitro suggest that cMoPs are direct GMP progeny.

However, it is possible that Ms4a3 expression could be acquired in between MDP and monocyte stages. To test this, we sorted tdTomato−MDPs and tdTomato+ and tdTomato−GMPs and cultured in vitro for 4 days. We observed that tdTomato+ and tdTomato−GMPs gave rise to exactly the same populations in culture, including monocytes, neutrophils, and some macrophages, while MDPs gave rise to DCs, monocytes, and macrophages (Figures S5E and S6F). tdTomato−GMPs and progenies rapidly became tdTomato+ (79.8% ± 0.20% at day 1 and 98.6% ± 0.25% at day 4), while the tdTomato labeling in tdTomato−MDP culture increased slowly to reach 30.6% ± 1.65% at day 4 (Figure 5C). MDP progeny included monocytes and DCs (CD11c−MHCIIhi); among them, CD11c+ cells were not labeled (1.00% ± 0.14%), while monocytes were highly labeled (75.1% ± 2.6%) (Figure 5D). These data suggest that Ms4a3 also labeled MDP-derived monocytes.

To further confirm these findings in vivo, we sorted tdTomato−GMPs, tdTomato+ MDPs, and tdTomato−MDPs from Ms4a3Cre-RosaTdt mice and adoptively transferred them into CD45.1 recipients. Consistent with the in vitro culture observations, tdTomato−GMPs rapidly became tdTomato+ and gave rise to monocytes and neutrophils but not to MDPs nor DCs (CD11c+) (Figures 5E–5G and S6G). In contrast, MDPs gave rise to CD11c−MHCII− (likely to be DC precursors) and monocytes, but not to neutrophils (Figures 5E, 5F, and S6G). Importantly, GMP-derived monocytes were fully labeled (100%). MDP-derived monocytes were also highly labeled (85.3% ± 3.60%), while MDP-derived CD11c−MHCII− cells were not (1.51% ± 0.82%) (Figure 5H). Both tdTomato+ MDPs and tdTomato− GMPs gave rise to monocytes through an identifiable cMoP (CD117+CD16/32−CD135−CD34+CD115+Ly6C+) at stage 1 but more clearly at day 2 (Figure 5E), while MDPs seem to give rise to monocytes through a different pathway, as we did not identify a cMoP intermediate in these experiments. MDPs lost CD117 expression from day 1, becoming tdTomato+CD117−CD16/32cells that differentiate into tdTomato+CD16/32CD115+Ly6C+ monocytes within 4 days (Figure 5E; data not shown). In summary, the use of Ms4a3Cre-RosaTdt model revealed that MDPs do not arise from GMPs and give rise to monocytes without going through a cMoP stage as commonly accepted.

**Monocytes Do Not Contribute Significantly to Tissue DCs with Age**

To determine whether tdTomato labeling fluctuates with age, we analyzed peripheral blood cells and splenic DCs from Ms4a3Cre-RosaTdt mice from birth up to 36 weeks. In the peripheral blood, we detected stable and efficient labeling of monocytes and neutrophils (Figures 6A and S7A) and no labeling of lymphocytes (Figure S7A). In the spleen, we found very low, but stable, cDC labeling and no pDC labeling (Figure S7B). Using a gating strategy previously proposed to identify cDCs in murine tissues (Guilliams et al., 2016), we also showed that tissue DCs exhibited very low labeling at 4 weeks (Figure S7C). When investigating whether monocyte-derived cells could accumulate in DC populations with age, we found slightly higher labeling of cDC2 at 12 weeks (Figure S7D). Identifying cDC2s is notoriously less straightforward than cDC1s, as cDC2-specific markers allowing unambiguous identification have not been formally identified. This population may therefore suffer from possible contamination by monocyte-derived cells. Whether such increase reflects a true contribution or contamination of monocytes to the DC pool remains to be investigated. This finding highlights that care must be taken to exclude monocyte-derived cells when identifying cDC2s in non-lymphoid tissues and that our model may help to address this issue in the future.

**The Contribution of Monocytes to RTMs Differs under Steady State between Organs and Shows a Tissue-Specific Gender Bias**

To investigate the kinetics of RTM replenishment by monocytes in Ms4a3Cre-RosaTdt mice, we analyzed the tdTomato labeling in RTMs in various tissues (brain, epidermis, liver, lung, peritoneal cavity, kidney, spleen, colon, small intestine, and dermis) at different ages (postnatal 0, 2 weeks, 4 weeks, 8 weeks, 20 weeks, 36 weeks). Of note, the labeling of blood monocytes was very efficient from birth (Figure 6A), whereas the labeling of tissue RTMs was negligible in newborn mice (Figures 6B–6E). This finding suggests that our fate-mapping model does not label embryonic macrophages or their precursors during development. In accordance with published reports, we found no postnatal changes in tdTomato labeling in brain microglia, epidermal LCs, or liver KCs during steady-state development (Figure 6B); conversely, tdTomato labeling in macrophages slowly increased with age in the lung, spleen, peritoneal cavity, and kidney (Figure 6C). As expected (Bain et al., 2016), we observed a rapid increase in tdTomato labeling in macrophages in the colon and small intestine over the first 8 weeks after birth, with a faster rate in the colon than in the small intestine (Figure 6D). Also in accordance with a previous report (Tamoutounour et al., 2013), dermal MHCII+ macrophages were replaced faster than MHCII− macrophages (Figure 6E). We confirmed
Figure 5. GMPs Do Not Give Rise to MDPs In Vitro and In Vivo
(A) tdTomato<sup>+</sup> GMPs (orange) and tdTomato<sup>−</sup> MDPs (blue) were cultured in vitro for 2 days, and production of MDPs, DCs, cMoPs, and monocytes was analyzed by flow cytometry. Experiment was repeated more than three times.

(B) Production of DCs, monocytes, neutrophils, and macrophages by indicated progenitors were analyzed by flow cytometry at day 4 of in vitro culture (n = 4). Experiment was repeated more than three times. Error bars, SEM.

(C) tdTomato labeling kinetics of indicated progenitor populations cultured in vitro as described in (A). Experiment was repeated twice with four replicates in each experiment. Error bars, SEM.

(D) tdTomato labeling in tdTomato<sup>−</sup> MDP-derived DCs, tdTomato<sup>−</sup> MDP-derived monocytes, and tdTomato<sup>−</sup> GMP-derived monocytes at day 4 of culture. Error bars, SEM.

(E) 10,000–20,000 tdTomato<sup>−</sup> GMPs (orange) and tdTomato<sup>−</sup> MDPs (blue) were adoptively transferred i.v. into non-irradiated CD45.1 recipient mice. The production of MDPs, DCs, cMoPs, and monocytes in the BM were analyzed by flow cytometry at day 2.

(F) Production of DCs, monocytes, and neutrophils by indicated progenitors was analyzed by flow cytometry at day 4 after adoptive transfer (n = 4). Error bars, SEM.

(G) tdTomato labeling kinetics of indicated progenitor populations in vivo as described in (E). Experiment was repeated twice. Error bars, SEM.

(H) tdTomato labeling in tdTomato<sup>−</sup> MDP-derived DCs, tdTomato<sup>−</sup> MDP-derived monocytes, and GMP-derived monocytes at day 4 of in vivo adoptive transfer. Experiment was repeated three times. Error bars, SEM.

See also Figure S6.
our flow-cytometry-based observations (Figure 6F) by immunofluorescence analysis of the corresponding tissues (Figure 6G). Most liver F4/80+ cells (92% ± 0.15%) likely to be KCs were tdTomato+, in agreement with their embryonic origin, while most of the gut (78% ± 1.24%) and dermal (70% ± 2.75%) F4/80+ macrophages were tdTomato+, underlining their derivation from tdTomato+ monocytes (Figures 6G and 6H). In a parallel comparison to our models, we analyzed the labeling of RTMs in Cx3cr1-Cre-Rosa<sup>Tdt</sup> and Cx3cr1<sup>CreERT2</sup>-Rosa<sup>Tdt</sup>. In the Cx3cr1<sup>CreERT2</sup>-Rosa<sup>Tdt</sup>, all RTMs were labeled, including the RTMs negative for Cx3cr1 expression (LCs, KCs, AMs, splenic macrophages, and some dermal macrophages), reflecting the contribution of Cx3cr1<sup>+</sup> embryonic precursors (Figure S7E). In Cx3cr1<sup>CreERT2</sup>-Rosa<sup>Tdt</sup> mice, as predicted, microglia, gut macrophages, and kidney macrophages were fully labeled; other RTMs were either not labeled or labeled to a lesser extent based on their selective expression of Cx3cr1 (Figure S7F).

To formally exclude the intrinsic expression of Ms4a3 in RTMs that could contribute to their labeling in the constitutive Ms4a3<sup>Cre</sup>-Rosa<sup>Tdt</sup> fate-mapping model, we took advantage of the Ms4a3<sup>CreERT2</sup>-Rosa<sup>Tdt</sup>-inducible model. Mice were treated with four injections of tamoxifen i.p. or gavage (for peritoneal macrophage analysis), and RTM labeling was analyzed at day 7. While blood monocytes were fully labeled (Figure 3K), only gut and dermal macrophages exhibited minor tdTomato labeling (Figure 6I). Such low labeling, rather than expression of Ms4a3 by macrophages, likely reflects the recent recruitment and differentiation from monocytes within the experimental time window of 7 days. Thus, we gave one injection of tamoxifen to Ms4a3<sup>CreERT2</sup>-Rosa<sup>Tdt</sup> mice and analyzed the labeling 24 h after. BM Ly6<sup>CH</sup> monocytes were fully labeled (91.4% ± 1.36%), while blood Ly6<sup>CH</sup> monocytes were lowly labeled (13.8% ± 1.82%) (Figure 6J). Importantly, no tdTomato labeling was detected in gut or dermal macrophages (Figure 6J). Combined with the Ms4a3<sup>Tdt</sup> reporter mouse data where no reporter signal was detected (Figures S4A–S4I), we conclude that RTMs do not express Ms4a3 and that the tdTomato+ labeling in RTMs accurately reflects blood monocyte contribution.

To investigate the gender bias in RTM renewal, we compared the labeling of various RTM populations in males and females at 8 weeks and 20 weeks (Figures 6K and 6L). At 8 weeks, among the RTM populations analyzed, only peritoneal macrophages showed a significant difference between males and females (Figure 6K). At 20 weeks, however, we observed that peritoneal macrophages and kidney macrophages significantly differed between males and females (Figure 6L), consistent with a previous report (Bain et al., 2016). In summary, we confirmed using our fate-mapping model that the contribution of monocytes to RTMs differed between organs under steady-state conditions but also by gender in peritoneal and kidney macrophages.

**Monocyte Contribution to RTMs during Inflammation Is Model Dependent**

Inflammatory stimuli often induce monocyte recruitment; these monocytes might potentially contribute to RTMs upon the resolution of inflammation. To investigate the contribution of circulating monocytes to RTMs during inflammation, we established various experimental scenarios of inflammation, including (1) thioglycollate-induced peritonitis, (2) IL-4; anti-IL-4 complex (IL-4c) stimulation, (3) lipopolysaccharide (LPS)-induced peritonitis, (4) LPS and cytokine guanine dinucleotide (CpG) lung stimulation, and (5) clodronate-loaded liposome-mediated macrophage depletion.

In the thioglycollate-induced peritonitis model, we observed a marked decrease in the number of peritoneal macrophages at 12 h, which was concomitant with an increase in the relative number of DAPI<sup>+</sup> peritoneal macrophages, suggestive of induced peritoneal macrophage cell death (Figures 7A, S7G, and S7H). From day 3, tdTomato labeling of peritoneal macrophages was significantly higher (74.1%–92.6%) compared to the steady-state control (16.8% ± 3.478%), suggesting replacement by monocytes (Figure 7B). To formally exclude the intrinsic expression of Ms4a3 in RTMs during this inflammatory challenge, we sorted peritoneal macrophages at different time points after thioglycollate administration and performed RNA-seq. We did not detect any significant level of Ms4a3 expression in peritoneal macrophages at any time point tested (Figure S7I). We also performed a similar challenge in the Ms4a3<sup>CreERT2</sup>-Rosa<sup>Tdt</sup> mice after tamoxifen gavage for 4 days and 3 days chase. At day 7, just before induction of thioglycollate-induced peritonitis,
we analyzed the peripheral blood and peritoneal macrophages. Blood Ly6C<sup>hi</sup> monocytes were highly labeled (93.7% ± 1.14%), while peritoneal macrophages were not (0.03% ± 0.01%) (Figure S7J). We then analyzed the same populations 72 h after. Blood Ly6C<sup>hi</sup> monocytes labeling decreased (51.2% ± 5.75%), while peritoneal macrophages were highly labeled (87.3% ± 1.18%), suggesting that they arise from monocytes (Figure S7J). Altogether, we conclude that peritoneal macrophages do not intrinsically express Ms4a3 and that tdTomato labeling after thioglycolate-induced peritonitis can be attributed to recruited monocytes.

Next, we used IL-4c to induce local macrophage proliferation that is known to occur without a circulating monocyte contribution (Jenkins et al., 2011) (Figures 7C and S7K). As expected, we observed a 4-fold increase in the number of peritoneal macrophages (Figures 7D and 7E), which was not associated with any changes in tdTomato labeling (Figure 7F). This confirmed the numbers of peritoneal macrophages increased through local proliferation of all macrophages irrespective of origin, rather than the recruitment of blood monocytes.

Finally, we induced peritonitis in Ms4a3<sup>C<sup>cre</sup>-Rosa<sup>Tdt</sup></sup> mice by LPS i.p. injection. We observed no changes in tdTomato labeling in peritoneal macrophages at any time point, indicating no monocyte contribution to peritoneal macrophages (Figure S7L). It is important to note that in this condition, we did not observe any peritoneal macrophage cell death (Figure S7M). We made similar observations in other organs under inflammation. In LPS- and CpG-induced lung inflammation, we observed no differences in tdTomato labeling of AMs between treated mice and controls despite observing significant monocyte recruitment (Figures 7G and S7N). Again, no significant AM cell death was observed (Figure S7O). In contrast, in the clodronate-induced RTM depletion model, almost all macrophages in the spleen were depleted at day 1 (Figure 7H). By 3 weeks post-depletion, the repopulated macrophages were tdTomato<sup>+</sup> (control versus clodronate, 19.9% ± 0.56% versus 54.3% ± 1.70% and 80.6% ± 1.38%) (Figures 7I, S7P, and S7Q).

Taken together, our Ms4a3<sup>C<sup>cre</sup>-Rosa<sup>Tdt</sup></sup> and Ms4a3<sup>C<sup>cre</sup>-ER<sup>DT2</sup>-Rosa<sup>Tdt</sup></sup> fate-mapping models show the contribution of monocytes to RTMs under different inflammatory settings. Our data suggest that monocytes can fill the empty niche left by depleted macrophages and develop into monocyte-derived macrophages.

**DISCUSSION**

The classical model of hematopoiesis proposes that MDPs arise from GMPs and give rise to monocytes through a cMoP stage (Ginhoux and Jung, 2014; Guilliams et al., 2018; Terry and Miller, 2014). In a recent study, Yanez et al. showed that MDPs arise directly from CMPs independently of GMPs and that GMPs and MDPs give rise to monocytes via MPs and cMoPs, respectively (Yanez et al., 2017), although no phenotypic difference between MPs and cMoPs was reported. Our Ms4a3<sup>C<sup>cre</sup>-Rosa<sup>Tdt</sup></sup> model labeled GMPs, cMoPs, granulocytes, and monocytes but not MDPs or DCs, providing strong evidence that MDPs do not arise from GMPs as proposed by Yanez et al. Here, we found that MDPs do not give rise to cMoPs and give rise to monocytes and DCs in vitro and in vivo through a yet-undescribed tdTomato<sup>+</sup> CD117<sup>+</sup> CD16/32<sup>+</sup> stage. The nature of such population and its relationship to cMoPs and CDPs remains to be investigated. These results show that monocytes are generated through two distinct cellular pathways defined by complementary oligopotent progenitor populations and raise the question of the nature of GMP- versus MDP-derived monocytes and their physiological relevance in steady state and in inflammation. Furthermore, the existence of the MDP population as an independent and distinct stage of myelopoiesis could be challenged as already discussed by Sathe et al. (2014). Nevertheless, since both GMPs and MDPs gave rise to tdTomato<sup>+</sup> monocytes, it is not yet possible in our Ms4a3<sup>C<sup>cre</sup>-Rosa<sup>Tdt</sup></sup> model to discriminate between these two ontogenic pathways. Hence, our Ms4a3 fate mapper traces monocytes without discriminating their origins. Future studies including fate-mapping models of GMPs or MDPs will help to clarify these questions and help to identify the intermediate stages between MDPs and their monocyte progeny.

Using the Ms4a3<sup>C<sup>cre</sup>-Rosa<sup>Tdt</sup></sup> model, we systematically assessed the contribution of monocytes to RTMs under steady-state conditions. Our data are concordant with previously published reports, showing that some RTMs have no monocyte contribution (microglia, KCs, and LCs) and that others exhibit a tissue-specific monocyte contribution, as previously proposed (Ginhoux and Guilliams, 2016). We also observed fast (gut and dermis) versus slow (kidney, spleen, and peritoneum) RTM replacement. We also confirmed that in the dermis, MHCI<sup>+</sup> macrophages are replaced at a faster rate than MHCI<sup>−</sup> macrophages.

**Figure 7. Monocyte Contribution to RTMs during Inflammation Is Model Dependent**

(A) Flow cytometric analysis of peritoneal lavage from Ms4a3<sup>C<sup>cre</sup>-Rosa<sup>Tdt</sup></sup> mice at the indicated time points after i.p. injection of thioglycollate.

(B) tdTomato labeling in peritoneal macrophages at the indicated time points after i.p. thioglycollate injection. The data are representative of two independent experiments with 5–8 female mice per group. Error bars, SEM.

(C) Experimental protocol. Mice were injected with IL-4c on days 0 and 2 and analyzed on day 4.

(D) Flow cytometric analysis of peritoneal macrophages at the indicated time points after i.p. thioglycollate injection. The data are representative of two independent experiments with 5–8 female mice per group. Error bars, SEM.

(E) Absolute numbers of peritoneal macrophages in the peritoneal lavage isolated from female mice injected with IL-4c or PBS control. The data are representative of two independent experiments with 5–8 female mice per group. Error bars, SEM.

(F) tdTomato labeling in peritoneal macrophages from mice injected with IL-4c or PBS, n = 4–9 female mice per group. Error bars, SEM.

(G) Flow cytometric analysis of lung AMs in Ms4a3<sup>C<sup>cre</sup>-Rosa<sup>Tdt</sup></sup> mice injected with IL-4c or PBS control. Error bars, SEM.

(H) Flow cytometric analysis of lung AMs in Ms4a3<sup>C<sup>cre</sup>-Rosa<sup>Tdt</sup></sup> mice treated with 10 μg LPS or 50 μg CpG i.n.; tdTomato labeling of AMs was analyzed at the indicated time points, n = 3–4 mice for each time point. Error bars, SEM.

(I) tdTomato labeling in splenic macrophages from Ms4a3<sup>C<sup>cre</sup>-Rosa<sup>Tdt</sup></sup> mice after macrophage depletion by clodronate liposome i.v. injection, n = 4–5 for each group. Statistical significance is indicated by **p < 0.001; ns, not significant. Error bars, SEM.

See also Figure S7.
(Tamoutounour et al., 2013). These results highlight that within tissues, subpopulations of macrophages coexist with a unique homeostatic regime. The mechanisms underlying this heterogeneity could be attributed to differences in the environmental niches within the tissue or at the sub-tissular level. In agreement with this hypothesis, we recently showed that two independent monocyte-derived RTM populations coexist across tissues with distinct functional profiles in unique sub-tissular niches: a Lyve1MHCII+CX3CR1+ population that is mostly found surrounding the nerves and a Lyve1MHCII+CX3CR1− population that is often closely associated with blood vessels across tissues (Chakarov et al., 2019).

None of the RTM populations that showed a significant contribution from monocytes with time—in particular dermal MHCII+ and gut (of which most are MHCII+) macrophages—exhibited total replacement by monocytes. The level of replacement reached its asymptote, for example ~12 weeks for dermal MHCII+ and gut macrophages, and no further increase was observed thereafter. These results suggest that at its asymptotic phase, the tissue likely reaches an equilibrium of monocyte recruitment, proliferation, and survival or death between adulthood-derived RTMs and proliferation and survival or death of embryonic-derived RTMs. Future studies are warranted to formally establish the exact contribution of monocyte recruitment, proliferation, and survival or death at these later time points in each RTM population. Furthermore, it should be ascertained whether such equilibrium is not the result of heterogeneous populations in origin residing in different sub-tissular localizations, for each tissue macrophage population or subpopulation studied. Nevertheless, for more defined populations of macrophages with a known niche, our observations suggest that tissues reach equilibrium in terms of macrophage homeostasis after 12 weeks. This equilibrium might simply reflect a sign of tissue maturity in terms of niche availability—a speculation that would be in agreement with the notion that macrophage homeostasis is controlled by niche access or availability (Guilliams and Scott, 2017). The implications of these ideas are important: they suggest that if tissue maturity in terms of macrophage content is not reached beyond 20 weeks, the most commonly chosen 6–8-week experimental time point used as a surrogate of adulthood may be premature.

Using our Ms4a3Cre,RosaTdT mouse model, we also found that the contribution of monocytes to macrophages differs according to sex and the challenge used to induce inflammation. These results highlight that the mechanisms behind different RTM renewal patterns are not fully understood and are likely controlled by the microenvironment, age, sex, and/or other factors such as the microbiome or diet; these mechanisms can now be readily tested by the scientific community using our models. In addition, we found that under inflammatory conditions that deplete RTMs (such as thioglycollate-induced peritonitis), monocytes infiltrate the tissue and replenish the macrophage population, while under inflammatory conditions that do not deplete RTMs (such as LPS-induced and CpG-induced lung inflammation and LPS-induced peritonitis), we did not observe a contribution of monocytes to RTMs. These observations again support the hypothesis that monocyte contribution to RTMs is subject to niche access and availability (Guilliams and Scott, 2017).

In conclusion, Ms4a3Cre,TdT reporter, and Ms4a3Cre,R and Ms4a3CreERT2 fate-mapping models permit the precise and faithful identification of monocytes and monocyte-derived cells in any condition and disentangle monocyte-derived macrophages from embryonic RTMs. These models will be critical to improve our understanding of the function of embryonic RTMs and BM-derived macrophages in homeostasis and inflammation and other models of disease including infection, cancer, and metabolic diseases.

LIMITATIONS

Although Ms4a3-based models clearly fate map monocytes and granulocytes, they could not distinguish monocytes with different origins (GMPs versus MDPs). Furthermore, although we did not observe any intrinsic labeling in RTMs in steady-state or inflammation settings tested here, we cannot formally exclude that few rare RTMs could start to express Ms4a3 in certain conditions not tested here, raising the need for complementary models that fate map only embryonic macrophages but not monocyte-derived macrophages.

STAR METHODS

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

- KEY RESOURCES TABLE
- LEAD CONTACT AND MATERIALS AVAILABILITY
- EXPERIMENTAL MODEL AND SUBJECT DETAILS
  - Animals
- METHOD DETAILS
  - Single cell RNA-sequencing (scRNA-seq)
  - scRNA-seq data analysis
  - Quantitative Real-Time PCR (qRT-PCR)
  - Tissue preparation for flow cytometry
  - Flow Cytometry
  - Cell Sorting
  - In vitro culture
  - In vivo transfer
  - Confocal microscopy
  - Tamoxifen induction
  - Inflammatory models
  - Bulk RNA Sequencing
  - Macrophage depletion with clodronate liposome
- QUANTIFICATION AND STATISTICAL ANALYSIS
  - Statistical analysis
- DATA AND CODE AVAILABILITY

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AUTHOR CONTRIBUTIONS

Zhaoyuan L., Y.G., S.C.B., I.K., X.C., A. Shin, W.H., R.J.D., and A. Schlitzer conducted the experiments; Zhaoyuan L., S.C.B., J.C., C.-A.D., B.S., and F.G. analyzed the data; Zhaoyuan L. and F.G. wrote the paper; H.W., Zhiduo L., and L.G.N. provided intellectual input; B.S. and F.G. supervised the project; F.G. conceptualized the study.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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REFERENCES


### METHODS

#### KEY RESOURCES TABLE

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(Continued on next page)
Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Florent Ginhoux (florent_ginhoux@immunol.a-star.edu.sg).

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| Collagenase type IV | Sigma | Cat# C5138 |
| DNase I | Roche | Cat# 1 0104159 001 |
| Dispase | GIBCO | Cat# 17105-041 |
| LPS-EB Vaccigene | InvivoGen | Cat# vac-3pelps |
| CpG | InvivoGen | Cat# ODN 1826 |
| Thioglycollate Medium Brewer Modified | BD | REF# 211716 |
| DAPI | ThermoFisher | Cat# D1306 |
| Recombinant mouse IL-4 | Peprotech | Cat# 214-14 |
| Clodronate Liposomes | Yeasen | Cat# 40337ES10 |
| Control Liposomes (PBS) | Yeasen | Cat# 40338ES10 |
| Tamoxifen | Sigma | Cat# T5648 |
| Trizol reagent | ThermoFisher | Cat# 15596026 |
| GlycoBlue Coprecipitant | ThermoFisher | Cat# AM9515 |

| SuperScript II Reverse Transcriptase | ThermoFisher | Cat# 18064014 |
| Power SYBR Green PCR Master Mix | ThermoFisher | Cat# 4387659 |
| Direct Lineage Cell Depletion Kit | Miltenyi | Cat# 130-110-470 |

| scRNA-seq data of monocyte/DC progenitors | This paper | GEO: GSE60783 |
| Bulk mRNA-seq data | This paper | NCBI BioProject SRA: PRJNA555467 |

| Mouse: Rosa26CdTomato | Madisen et al., 2010 | JAX 007914 |
| Mouse: Ms4a3Cre | This paper | N/A |
| Mouse: Ms4a3CreTomato | This paper | N/A |
| Mouse: Ms4a3CreERT2 | This paper | N/A |
| Mouse: Cx3cr1Cre | Jung et al., 2000 | JAX 005582 |
| Mouse: Cx3cr1CreErt2 | Yona et al., 2013 | JAX 025524 |

| qPCR primer sequences | This paper | See Method Details |
| Genotyping primer sequences | This paper | See Method Details |

| FlowJo V10 | FlowJo | https://www.flowjo.com |
| GraphPad Prism 6 | GraphPad Software | https://www.graphpad.com |
| Imaris | Bitplane | https://imaris.oxinst.com |
EXPERIMENTAL MODEL AND SUBJECT DETAILS

Animals
Ms4a3^{TdT}, Ms4a3^{Cre} and Ms4a3^{CreERT2} mice were generated at the Shanghai Model Organisms Center, Inc. Briefly, an Ires-tdTomato or Ires-Cre or Ires-CreERT2 gene fusion was inserted into the 3’ un-translated region (3’UTR) of the Ms4a3 gene by CRISPR-Cas9 technique in C57BL/6 zygotes (Ms4a3^{TdT} and Ms4a3^{Cre}) or by homologous recombination in ES cells (Ms4a3^{CreERT2}). To eliminate off-target effects, knock-in mice were then backcrossed onto a C57BL/6 background for three generations. All these three mouse strains were genotyped by PCR using the following primers:

Common forward primer 5’- AGAGAAATCATCAGGCGAAGAT-3’;
Mutant reverse primer 5’- TTGGCGAGAGGGAAGAC-3’ (412 bp fragment);
Wild-type reverse primer 5’-GAAAAGGGAACACGGCAAGAT-3’ (517 bp fragment).

Rosa26^{tdTomato} reporter mice have been previously described (Madisen et al., 2010). Cx3cr1^{flopx}, Cx3cr1^{Cre} and Cx3cr1^{CreERT2} mice have been previously described (Jung et al., 2000; Yona et al., 2013). All mice were bred in a specific pathogen-free animal facility at the Shanghai Jiao Tong University School of Medicine. All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of Shanghai Jiao Tong University School of Medicine and were performed in compliance with the University’s guidelines for the care and use of laboratory animals.

METHOD DETAILS

Single cell RNA-sequencing (scRNA-seq)
Cell populations (blood Ly6C^- monocytes, BM Ly6C^- monocytes and BM cMoPs) were isolated by FACS and diluted to a final concentration range of 250–400 cells/μL. The cells were then loaded onto C1 integrated fluidic circuits (5-10-μm chip) for cell lysis, reverse transcription with oligo (dT) primers and cDNA amplification on a C1 Single-cell Auto Prep System, according to the manufacturer’s mRNA-seq protocol (Fluidigm). Array control RNA spikes were used (1, 4 and 7) (PN AM1781) according to the manufacturer’s protocol (Ambion). The cDNA generated from single cells was quantified with a Quantit PicoGreen dsDNA Assay Kit (PN P11496; Life Technologies), and the quality was checked using High Sensitivity DNA Reagents (PN 5067-4626), according to the manufacturer’s instructions (Agilent Technologies). Only cells with high-quality cDNA were processed for subsequent library preparation. A Nextera XT Kit (PN FC-131-1096; Illumina) with dual indices (PN FC-131-1002; Illumina) was used to prepare single-cell multiplexed libraries, which were sequenced as 51-bp single-end reads on an Illumina HiSeq 2000 platform. Single-end reads were mapped to the mm9 reference genome (NCBI assembly of the mouse genome).

scRNA-seq data analysis
CMap analysis is an extension of the GSEA algorithm (provided by the Broad Institute) in which ‘enrichment’ of a gene set (signature genes) in another gene set can be measured. CMap scores are scaled, dimensionless quantities that indicate the degree of enrichment or ‘closeness’ of one assessed cell subset to another. Monocyte and DC signature genes were identified from both the literature and our transcriptomic data, and were used as signature genes for the respective populations for CMap analysis of each single cell. The ‘enrichment’ of gene sets was tested with 1,000 permutations. Cells with a gene-expression profile that significantly correlated with signature genes were selected by a P value of < 0.05 after 1,000 permutations. CMap scores were scaled to a range of –1 to 1. Cells with a positive CMap score were denoted as monocytes or monocyte primed cells, while cells with a negative CMap score were denoted as DCs or DC-primed cells. DEGs between monocyte-primed progenitors versus DC-primed progenitors at the BM MDP, CD115^+ monocytes, BM Ly6C^- monocytes and BM cMoPs stages were identified using the Seurat R package. The bimodal likelihood-ratio test for single cell gene expression was used for DEG analysis, and genes with adjusted p values < 0.05 were identified as being differentially expressed.

Quantitative Real-Time PCR (qRT-PCR)
Total RNA was isolated from sorted cells using TRizol reagent (Invitrogen), according to the manufacturer’s protocol. Glycoblue (Invitrogen) was added as a co-precipitant when handling < 10^6 cells. cDNA was synthesized using an M-MLV First-Strand Synthesis Kit (Invitrogen, C28025-021) with oligo (dT) primers. qRT-PCR was performed using FastStart Universal SYBR Green Master with Rox (Applied Biosystems) on a ViIA 7 Real-Time PCR system (Applied Biosystems). The following primers were used for qRT-PCR:

Ms4a3 forward primer 5’- GTGGTCTCTTATATAGCCCTT-3’;
Ms4a3 reverse primer 5’- ACAGTGGTAGCCTGTGTAAGA-3’;
tdTomato forward primer 5’- CCTGTCCCTGGGCGATGG-3’;
tdTomato reverse primer 5’- TAGATGACGGCCATGTTGTTG-3’;
Gapdh forward primer 5’- AGGTCGGTGTGAACGGATTTG-3’;
Gapdh reverse primer 5’- TGATGACGGCCATGTTGAGGTCA-3’.

All data were normalized to Gapdh quantified in parallel amplification reactions.
**Tissue preparation for flow cytometry**

Blood was collected by cardiac puncture from terminally anaesthetized mice; the mice were then euthanized by cervical dislocation. Peritoneal lavage was obtained by injecting 5 mL PBS containing 2 mM EDTA into the peritoneal cavity, and the washout was collected. The mice were then perfused with PBS via the left ventricle. The spleen was harvested and homogenized into a single-cell suspension using a 70 μm cell strainer and syringe plungers, then lysed in ACK lysis buffer (155 mM NH₄Cl, 10 mM KHCO₃, 0.1 mM EDTA) and again passed through a 70 μm cell strainer.

To obtain microglia, the brain was cut into small pieces and digested with 0.2 mg/mL collagenase type IV (C5138, Sigma) and 0.05 mg/mL DNase I (Roche) in RPMI640 medium with 10% FCS at 37°C for 60 min. The digested suspension was homogenized with a syringe with a 1.2 mm inner diameter needle. The brain-cell suspension was separated by 40%/80% layered Percoll (GE Healthcare) gradient centrifugation at 1,578 x g for 20 min at room temperature with low acceleration and no brake. The middle interface layer was collected. For newborn mice, the digested brain suspension was used for staining without Percoll separation.

For skin preparation (Tamoutounour et al., 2013), ears were split between the dorsal and ventral sections, and digested in dispase solution (GIBCO) at 37°C for 90 min. The dermis and epidermis were separated and further digested as described for brain tissues. The dermis and epidermis were disrupted into single-cell suspensions using a syringe with a 1.2 mm inner diameter needle and passed through a 70 μm cell strainer.

For adult lung, liver and kidney tissues and newborn mouse tissues, tissues were cut into small pieces and digested and homogenized as described for brain preparations. Red blood cells were lysed in ACK lysis buffer.

For intestine preparation (Bain et al., 2014), the colon and small intestine were removed and washed in PBS, and the fat tissue and Peyer’s patches in the small intestine were removed. The intestines were opened longitudinally, cut into 0.5 cm sections and washed four times with PBS. After washing, 12.5 mL fresh calcium/magnesium-free PBS containing 5 mM EDTA and 2 mM DTT was added and the tube was incubated at 37°C with agitation for 20 min to detach the epithelial cells. The epithelial sheet was removed by vigorous shaking and the remaining tissue was washed twice with PBS, cut into small pieces and then digested and homogenized as described for brain preparations.

**Flow Cytometry**

For BM progenitor analysis, BM cells were stained with APC-Cy7 conjugated anti-CD16/32 (clone 93; Biolegend) at 4°C for 15 min, and then stained with other antibodies used for flow cytometry can be found in the Key Resources Table. PE-Cy7-conjugated streptavidin was used to detect biotin-labeled CD135 (clone A2F10; eBioscience). Lineage markers used in the BM progenitor analysis included CD3e, CD19, CD49b, Ly6G, Ter-119, B220, CD11c and CD11b. For flow cytometry of other samples, nonspecific antibody binding to cells was blocked by incubation with an anti-CD16/32 antibody (clone 2G8; BD Biosciences) at 4°C for 15 min, and the cells were stained with fluorophore-conjugated or biotin-conjugated antibodies at 4°C for 25 min. Cells were maintained at 4°C and analyzed on a BD Fortessa X20 or Symphony (BD Biosciences). Data were analyzed in FlowJo (FlowJo LLC). tSNE analysis was calculated with all the markers used for flow cytometry, except tdTomato.

**Cell Sorting**

For peripheral blood, splenic DC and RTM cell sorting, nonspecific antibody binding to cells was blocked by incubating cells with an anti-CD16/32 antibody (clone 2.4G2; BD Biosciences) at 4°C for 15 min. The cells were then stained with fluorophore-conjugated antibodies (Key Resources Table) at 4°C for 25 min. FACS was performed on a BD FACS Aria III (BD Biosciences) to achieve > 95% purity. Dead cells were excluded by DAPI (Invitrogen) staining.

For BM progenitor cell sorting, BM cells from the tibia and femur were used and lineage cells were depleted with a Direct Lineage Depletion Kit (Lin: CD5, CD11b, CD45R [B220], Anti-Gr-1 [Ly-6G/C], 7-4, and Ter-119; Miltenyi). Lin− cells were stained with APC-Cy7 conjugated with an anti-CD16/32 antibody and incubated at 4°C for 15 min before staining with other antibodies and secondary PE-Cy7 conjugated streptavidin at 4°C for 25 min. FACS was performed on a BD FACS Aria III (BD Biosciences) to achieve > 95% purity.

**In vitro culture**

Indicated BM progenitor cells were sorted from pooled BM of CD45.2 Ms4a3Cre-RosaTdT mice and mixed with 500,000 BM cells from CD45.1 mice, and cultured in vitro in media (RPMI with 10% FCS and 1% penicillin-streptomycin) supplemented with M-CSF (50 ng/mL; Peprotech). Cells were harvested and analyzed by flow cytometry at the indicated time point.

**In vivo transfer**

Indicated BM progenitor cells were sorted from pooled BM of Ms4a3Cre-RosaTdT (CD45.2 background) mice. Progenitor cells were intravenously or intra-bone marrow transferred into non-irradiated CD45.1 recipient mice (10,000-20,000 cells/mouse). Donor derived cells in the BM (femurs and tibias), spleen and blood were analyzed at the indicated time point.

**Confocal microscopy**

Tissues were harvested and fixed overnight in fixation buffer containing 1% PFA. The tissues were then dehydrated in 30% sucrose before embedding in OCT freezing media (Sakura). Sections were cut to 10-μm on a Leica cryostat and blocked for 1 h at room temperature in blocking buffer containing 1% normal mouse serum, 1% BSA and 0.3% Triton X-100. The sections were stained with the...
indicated fluorophore-conjugated antibodies overnight at 4°C in a dark, humidified chamber. Antibodies for immunofluorescence can be found in the Key Resources Table. Images were captured under a Leica TCS SP8 laser confocal microscope (Leica).

**Tamoxifen induction**
Tamoxifen was prepared by dissolving in corn oil for a final concentration of 17.5 mg/mL and stored at −20°C. Adult Ms4a3CreERT2,RosaTdT and Cx3crlCreERT2-RosaTdT mice were given 100 μL (1.75 mg) tamoxifen solution once a day for 4 days by i.p. injection or by gavage as indicated.

**Inflammatory models**
For thioglycollate-induced sterile peritonitis, 1 mL of 4% sterile thioglycollate broth (BD Biosciences) was injected i.p. into 8-week-old female mice and then analyzed at the indicated time points. For long-acting IL-4 treatment, a mix of 5 μg recombinant mouse IL-4 (Peprotech) and 25 μg anti-IL-4 mAb (clone 11B11; BioXcell, NH) was incubated for 5 min on ice to form an IL-4:anti-IL-4 complex (IL-4c). IL-4c enables sustained and slow IL-4 release. Mice were then injected i.p. with IL-4c (containing 5 μg IL-4 and 25 μg anti-IL4), or PBS vehicle control on days 0 and 2. The peritoneal lavage was analyzed at the indicated time points. For LPS-induced peritonitis, 50 μg sterile LPS (InvivoGen) was injected i.p. and peritoneal lavage was analyzed at the indicated time points. For LPS and CpG-induced lung inflammation models, lightly anesthetized mice with isoflurane were instilled i.n. with 50 μL saline (vehicle control) or 50 μL saline containing 10 μg LPS (InvivoGen) or 50 μg CpG (InvivoGen). Mice were then analyzed at the indicated time points.

**Bulk RNA Sequencing**
Peritoneal macrophages and monocytes were sorted from control and thioglycollate treated mice at different time points. Total RNA was isolated from sorted cells using TRIzol reagent (Invitrogen), according to the manufacturer’s protocol. Total RNA was reverse transcribed with the SMART-seq2 protocol (Picelli et al., 2014). Samples were sequenced on an Illumina NextSeq 500 sequencer using pair-end 75 base pair reading. Sequencing data were aligned to the mouse reference genome (version mm10).

**Macrophage depletion with clodronate liposome**
Mice were injected i.v. with 200 μL clodronate liposome (Yeasen) or empty liposome control to deplete RTMs in the spleen. Tissues were harvested at the indicated time points for flow cytometry.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

**Statistical analysis**
The statistical analyses performed for each experiment are indicated in the figure legends. No statistical methods were used to predetermined the sample size. The experiments were not randomized. The investigators were not blinded to allocation during experiments and outcome assessment.

**DATA AND CODE AVAILABILITY**
The scRNA-seq dataset is deposited in the Genome Expression Omnibus under accession number GEO: GSE134523. The bulk RNA-seq data is deposited in the Sequence Read Archive (SRA) under accession number SRA: PRJNA555467.
Figure S1. Ms4a3 Is Specifically Expressed by Monocyte-Committed Progenitors, Related to Figure 1

(A) scRNA-seq workflow of BM cMoPs, BM monocytes and blood monocytes using Fluidigm C1 autoprep system.
(B) Sorting panel for blood Ly6C$^+$ monocytes.
(C) Sorting panel for BM cMoPs and BM Ly6C$^+$ monocytes. Lineage markers include CD3e, CD19, CD49b, Ly6G.
(D) Heatmap generated with the top 10 DEGs for each population.
(E) Ms4a3 expression profile in BM progenitors using ImmGen dataset.
(F) Ms4a3 expression profile in DCs and RTM populations using ImmGen dataset.
(G) Ms4a3 expression profile using BioGPS dataset.
(A) Sorting panel for BM CMPs, GMPs, GPs, cMoPs, Ly6C$^+$ monocytes, MDPs and CDPs. BM CMPs were defined as Lin$^-$Sca-1$^-$.CD117$^+$CD16/32$^-$CD34$^+$CD135$^+$CD115$^-$. GMPs as Lin$^-$Sca-1$^-$.CD117$^+$CD16/32$^+$CD34$^+$CD135$^+$Ly6C$^-$CD115$^-$. GPs as Lin$^-$Sca-1$^-$.CD117$^+$CD16/32$^+$CD34$^+$CD135$^+$Ly6C$^-$.CD115$^-$. cMoPs as Lin$^-$Sca-1$^-$.CD117$^+$CD16/32$^+$CD34$^+$CD135$^+$Ly6C$^+$CD115$^-$. BM monocytes as Lin$^-$Sca-1$^-$.CD117$^+$CD16/32$^+$CD34$^+$CD135$^+$Ly6C$^+$Ly6C$^-$.CD115$^-$. MDPs as Lin$^-$Sca-1$^-$.CD117$^+$CD16/32$^+$CD34$^+$CD135$^+$Ly6C$^+$Ly6C$^-$.CD115$^-$. CDPs as Lin$^-$Sca-1$^-$.CD117$^+$CD16/32$^+$CD34$^+$CD135$^+$Ly6C$^+$Ly6C$^-$.CD115$^-$. Lineage markers include CD3e, CD19, CD49b, Ly6G, Ter-119, B220, CD11c and CD11b.

(B) Sorting panel for myeloid lineage cells (basophils, neutrophils, eosinophils, and Ly6C$^+$ and Ly6C$^-$ monocytes) in peripheral blood.

(C) Sorting panel for lymphoid lineage cells (B cells, NK cells, CD4$^+$ T cells and CD8$^+$ T cells) in peripheral blood.

(D) Sorting panel for splenic cDCs (cDC1 and cDC2) and pDCs.

(E) Sorting panel for peritoneal macrophages.

(F) Sorting panel for lung AMs.

(G) Sorting panel for liver KCs.

(H) Sorting panel for gut macrophages.

(I) Sorting panel for epidermal LCs.

(J) Sorting panel for brain microglia cells.
Figure S3. Parallel Comparison of Ms4a3Tet and Cx3cr1GFP, Related to Figure 2

(A) Schematic of Ms4a3Tet mice. An Ires-tdTomato-pA cassette was inserted after the stop codon.

(B) Expression of Cx3cr1-GFP in the indicated BM progenitors in Cx3cr1GFP (filled gray) and WT (open) mice.

(legend continued on next page)
(C) Expression of Cx3cr1-GFP in the indicated cell types in peripheral blood of Cx3cr1<sup>fl/fl</sup> (filled gray) and WT (open) mice.

(D) tSNE plot shows the intensity of Cx3cr1-GFP in peripheral blood cells, the color indicates the expression intensity of GFP, red indicates high expression, blue indicates low expression.

(E) Expression of Cx3cr1-GFP in splenic cDCs and pDCs from Cx3cr1<sup>fl/fl</sup> (filled gray) and WT (open).

(F) Flow cytometric analysis of peripheral blood cells of Cx3cr1<sup>fl/fl</sup>;Ms4a3<sup>tdTomato</sup> mice. Differential expression of Cx3cr1-GFP and Ms4a3-tdTomato could be used to distinguish different myeloid populations.
Figure S4. Expression of Ms4a3-tdTomato and Cx3cr1-GFP in RTMs, Related to Figure 2
(A) Gating strategy of brain microglia (CD45<sup>int</sup>CD11b<sup>+</sup>F4/80<sup>−</sup>Ly6C<sup>−</sup>), histogram showing the intensity of tdTomato expression in microglia of 8-week-old and 6-month-old Ms4a3<sup>TdT</sup> (filled red) and WT mice (open), and Cx3cr1-GFP expression in 8-week-old Cx3cr1<sup>gfp</sup> (filled green) and WT mice (open).
(B) Gating strategy of epidermal LCs (CD45<sup>+</sup>Thy1<sup>−</sup>CD11b<sup>+</sup>F4/80<sup>+</sup>EpCAM<sup>+</sup>), histogram showing the intensity of tdTomato expression in LCs of 8-week-old and 6-month-old Ms4a3<sup>TdT</sup> (filled red) and WT mice (open), and Cx3cr1-GFP expression in 8-week-old Cx3cr1<sup>gfp</sup> (filled green) and WT mice (open).
(C) Gating strategy of liver KCs (CD45<sup>+</sup>Lin<sup>−</sup>CD11b<sup>+</sup>F4/80<sup>+</sup>Tim-4<sup>+</sup>), lineage markers include CD3e, CD19, CD49b, Ly6G. Histogram showing the intensity of tdTomato expression in KCs of 8-week-old and 6-month-old Ms4a3<sup>TdT</sup> (filled red) and WT mice (open), and Cx3cr1-GFP expression in 8-week-old Cx3cr1<sup>gfp</sup> (filled green) and WT mice (open).
(D) Gating strategy of AMs (CD45<sup>+</sup>SiglecF<sup>+</sup>CD11c<sup>+</sup>CD11b<sup>+</sup>Ly6C<sup>−</sup>), histogram showing the intensity of tdTomato expression in AMs of 8-week-old and 6-month-old Ms4a3<sup>TdT</sup> (filled red) and WT mice (open), and Cx3cr1-GFP expression in 8-week-old Cx3cr1<sup>gfp</sup> (filled green) and WT mice (open).
(E) Gating strategy of splenic macrophages (CD45<sup>+</sup>Lin<sup>−</sup>F4/80<sup>+</sup>), lineage markers include CD3e, CD19, CD49b, Ly6G. Histogram showing the intensity of tdTomato expression in splenic macrophages of 8-week-old and 6-month-old Ms4a3<sup>TdT</sup> (filled red) and WT mice (open), and Cx3cr1-GFP expression in 8-week-old Cx3cr1<sup>gfp</sup> (filled green) and WT mice (open).
(F) Gating strategy of peritoneal macrophages (CD45<sup>+</sup>CD11b<sup>+</sup>F4/80<sup>+</sup>), histogram showing the intensity of tdTomato expression in peritoneal macrophages of 8-week-old and 6-month-old Ms4a3<sup>TdT</sup> (filled red) and WT mice (open), and Cx3cr1-GFP expression in 8-week-old Cx3cr1<sup>gfp</sup> (filled green) and WT mice (open).
(G) Gating strategy of kidney macrophages (CD45<sup>+</sup>CD11b<sup>+</sup>F4/80<sup>+</sup>MHCII<sup>+</sup>), histogram showing the intensity of tdTomato expression in kidney macrophages of 8-week-old and 6-month-old Ms4a3<sup>TdT</sup> (filled red) and WT mice (open), and Cx3cr1-GFP expression in 8-week-old Cx3cr1<sup>gfp</sup> (filled green) and WT mice (open).
(H) Gating strategy of gut macrophages (CD45<sup>+</sup>SiglecF<sup>+</sup>Ly6G<sup>−</sup>CD11c<sup>+</sup>CD11b<sup>+</sup>CD64<sup>+</sup>Ly6C<sup>−</sup>MHCII<sup>+</sup>), histogram showing the intensity of tdTomato expression in gut macrophages of 8-week-old and 6-month-old Ms4a3<sup>TdT</sup> (filled red) and WT mice (open), and Cx3cr1-GFP expression in 8-week-old Cx3cr1<sup>gfp</sup> (filled green) and WT mice (open).
(I) Gating strategy of dermal MHCII<sup>+</sup> macrophages (CD45<sup>+</sup>CD11b<sup>+</sup>F4/80<sup>+</sup>MHCII<sup>+</sup>) and MHCII<sup>−</sup> macrophages (CD45<sup>+</sup>CD11b<sup>+</sup>F4/80<sup>+</sup>MHCII<sup>−</sup>), histogram showing the intensity of tdTomato expression in dermal macrophages of 8-week-old and 6-month-old Ms4a3<sup>TdT</sup> (filled red) and WT mice (open), and Cx3cr1-GFP expression in 8-week-old Cx3cr1<sup>gfp</sup> (filled green) and WT mice (open).
Figure S5. Parallel Comparison of Ms4a3-Based and Cx3cr1-Based Models, Related to Figure 3

(A) Schematic of Ms4a3Cre strategy. An Ires-Cre cassette was inserted after the stop codon. Ms4a3Cre mice were crossed with RosaTdT reporter mice. In Cre-expressing cells, the Stop signal was irreversibly removed and tdTomato expression was induced. (B) Flow cytometric analysis of tdTomato expression in CD45+ tissue cells in Ms4a3Cre-RosaTdT mice. Brain, epidermis, dermis, liver, lung, kidney, pancreas, heart, salivary gland, colon, small intestine and testis were analyzed. Experiments were repeated six times with 3-4 mice for each experiment. (C) Microscopic analysis showed dim tdTomato (red) expression in CD45+ cells in the testis of Ms4a3Cre-RosaTdT mice. Cyan is CD45 and green is F4/80. (D) tdTomato expression in CD45+ cells in testis of Ms4a3TdT reporter mice (filled gray) and WT mice (open black). (E) A Cx3Cr1Cre-based fate mapping model showed that Ly6Clo monocytes are derived from Ly6C hi monocytes (Yona et al., 2013); thus, labeling in Ly6Clo monocytes should not be less than the labeling observed in Ly6C hi monocytes. Our differential labeling pattern (95.1% versus 97.3%) suggested that Ly6C hi monocytes could either be a heterogeneous population, with a minor tdTomato+ fraction not arising from the Ly6C hi compartment, or that tdTomato+ cells contaminated the Ly6C lo monocyte gate. To refine the gating of the Ly6C lo monocyte population, we stained with the Ly6C lo monocyte marker, CD43 (Ingersoll et al., 2010; Yanez et al., 2017): refining the Ly6C lo CD43+ monocyte gate resulted in tdTomato Ly6C hi monocyte labeling that was identical to Ly6C hi monocyte labeling (97.3+/−0.30% versus 97.7+/−0.43%, respectively). tdTomato labeling in blood Ly6C hi, Ly6C lo and Ly6C lo CD43+ populations in CD115+ monocytes, n = 9. (F) tSNE plot shows the labeling of tdTomato in peripheral blood, the color indicates the expression intensity of tdTomato, red indicates high expression, blue indicates low expression. (G) tdTomato labeling in different lymphoid and myeloid lineages of Cx3cr1Cre-RosaTdT mice. The data are representative of 4 individual mice. The error bars represent SEM. (H) Schematic of Ms4a3CreERT2 mice. An Ires-CreERT2-pA cassette was inserted after the stop codon. (I) Experimental protocol. Mice were injected with tamoxifen for 4 successive days, and analyzed on day 7. (J) tSNE plot shows the labeling of Cx3cr1-GFP in peripheral blood of Cx3cr1CreERT2-RosaTdT mice, the color indicates the expression intensity of Cx3cr1, red indicates high expression, blue indicates low expression. (K) tdTomato labeling in different lymphoid and myeloid lineages of Cx3cr1CreERT2-RosaTdT mice induced with tamoxifen. The data are representative of 4 individual mice. The error bars represent SEM.
Figure S6. MDPs Do Not Arise from GMPs In Vitro and In Vivo, Related to Figure 4
(A) Analysis of tdTomato+ BM cells. Plots show the distribution of tdTomato+ cells across different progenitor populations. (B) Analysis of tdTomato− BM cells. Plots show the distribution of tdTomato− cells across different progenitor populations. (C) Flow cytometric analysis of MDP defined by Fogg et al., the Ms4a3-tdTomato+ cells in the MDP gate were further analyzed with the gating strategy used by Hettinger et al. to define cMoPs. (D) Gating strategy used by Yanez et al... (E) Gating strategy used by Hettinger et al... (F) Production of DCs, monocytes, neutrophils and macrophages by indicated progenitors were analyzed by flow cytometry at day 4 of in vitro culture. (G) Production of DCs, monocytes and neutrophils by indicated progenitors were analyzed by flow cytometry at day 4 after adoptive transfer.
Figure S7. Monocyte Contribution to RTMs in Steady State and Inflammation, Related to Figure 6 and 7

(A) Kinetics of tdTomato labeling in lineages in peripheral blood. tdTomato labeling in T cells, B cells, NK cells and neutrophils from Ms4a3Cre-RosaTdr mice was analyzed at different ages. n = 3-4 mice analyzed per time point. (B) Kinetics of tdTomato labeling in DCs in the spleen. tdTomato labeling in cDC1, cDC2 and pDCs from Ms4a3Cre-RosaTdr mice was analyzed at different ages. n = 3-4 mice analyzed per time point. (C) tdTomato labeling of cDC1 and cDC2 in different organs from 4-week-old Ms4a3Cre-RosaTdr mice. Experiments were repeated twice, and the data are representative of 4 mice. The error bars represent SEM. (D) tdTomato labeling of cDC1 and cDC2 in different organs from 12-week-old Ms4a3Cre-RosaTdr mice. Experiments were repeated twice, and the data are representative of 4 mice. The error bars represent SEM. (E) tdTomato labeling of cDC1 and cDC2 in different organs from 4-week-old Ms4a3Cre-RosaTdr mice, the data are representative of 4 individual mice. The error bars represent SEM. (F) tdTomato labeling in RTMs of Cx3cr1CreERT2-RosaTdr mice induced with 4 injections of tamoxifen and analyzed at day 7. The data are representative of 4 individual mice. The error bars represent SEM. (G) DAPI+ relative numbers in peritoneal macrophages were analyzed at the indicated time points after i.p. thioglycollate injection. Results from one experiment with 3 mice per time point are shown. (H) Relative numbers of peritoneal macrophages in CD45+ cells were analyzed at the indicated time points after i.p. thioglycollate injection. Results from one experiment with 3 mice per time point are shown. (I) Peritoneal macrophages were sorted from thioglycollate treated mice at different time points and monocytes sorted from peritoneal lavage from 6 h time point, the expression of Ms4a3 is shown, the y axis indicates TPMs of Ms4a3 in each group. (J) Ms4a3CreERT2-RosaTdr mice induced with 4 injections of tamoxifen and left for 3 days to washout tamoxifen, the mice were injected with thioglycollate (day 0) and analyzed on day 0 and day 3. tdTomato labeling in blood Ly6C+ monocytes and peritoneal macrophages are shown, the data are representative of 4 individual mice. The error bars represent SEM. (K) Images of spleen isolated from mice injected with IL-4c or PBS. (L) tdTomato labeling of peritoneal macrophages from Ms4a3Cre-RosaTdr mice injected with LPS was analyzed at the indicated time points, n = 3-4 for each group. (M) DAPI+ relative numbers in peritoneal macrophages were analyzed at the indicated time points after i.p. administration of LPS. Results from one experiment with 3 mice per time point are shown. (N) Relative numbers of neutrophils (yellow) and monocytes (blue) in live cells of the lung. Results from one experiment with three mice per time point. (O) DAPI+ relative numbers in AMs were analyzed at the indicated time points after i.n. administration of CpG or LPS. Results from one experiment with three mice per time point are shown. (P) Flow cytometric analysis of splenic macrophages from Ms4a3Cre-RosaTdr mice at the indicated time points after i.p. injection of 200 µL clodronate liposome and (Q) tdTomato labeling in splenic macrophages from Ms4a3Cre-RosaTdr after macrophage depletion by clodronate liposome i.p. injection, n = 5-6 for each group. Statistical significance is indicated by ***p < 0.001. The error bars represent SEM.