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Tissue-Resident Macrophages Self-Maintain Locally throughout Adult Life with Minimal Contribution from Circulating Monocytes

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SUMMARY

Despite accumulating evidence suggesting local self-maintenance of tissue macrophages in the steady state, the dogma remains that tissue macrophages derive from monocytes. Using parabiosis and fate-mapping approaches, we confirmed that monocytes do not show significant contribution to tissue macrophages in the steady state. Similarly, we found that after depletion of lung macrophages, the majority of repopulation occurred by stochastic cellular proliferation in situ in a macrophage colony-stimulating factor (M-Csf)- and granulocyte macrophage (GM)-CSF-dependent manner but independently of interleukin-4. We also found that after bone marrow transplantation, host macrophages retained the capacity to expand when the development of donor macrophages was compromised. Expansion of host macrophages was functional and prevented the development of alveolar proteinosis in mice transplanted with GM-Csf-receptor-deficient progenitors. Collectively, these results indicate that tissue-resident macrophages and circulating monocytes should be classified as mononuclear phagocyte lineages that are independently maintained in the steady state.

INTRODUCTION

A foundational dogma in immunology is the derivation of macrophages from monocytes, bolstering the conceptual framework that monocytes and macrophages can be grouped because they arise from the same developmental spectrum. However, this dogma was established with the mononuclear phagocyte system (MPS) theory (van Furth and Cohn, 1968) prior to the advent of flow cytometry and other more precise techniques that help to discriminate the individual components of the MPS. Macrophages broadly consist of two classes: tissue-resident macrophages and infiltrating macrophages. Examples of tissue-resident macrophages include liver Kupffer cells, microglia, and peritoneal, lung, splenic red pulp, and bone marrow (BM) macrophages, which by definition reside in and perform homeostatic functions in their respective tissues in the steady state

(Murray and Wynn, 2011). On the other hand, infiltrating macrophages are only found after an inciting pathology. It has become clear that classical monocytes (also known as "inflammatory" or Ly6C^{hi} monocytes) are the source of infiltrating macrophages found in pathological settings, such as cancer (Qian et al., 2011), atherosclerosis (Ingersoll et al., 2011), and metabolic disease (Chawla et al., 2011). However, monocyte contribution to tissue-resident macrophages is still controversial. In vivo labeling studies involving radioisotopes and radiation chimera experiments in the 1960s made immunologists confident that BM-derived cells, probably monocytes, replenish the tissue-resident macrophage compartment (van Furth and Cohn, 1968; Virolainen, 1968). These studies were supported by early parabiosis studies that reported monocyte contribution to peritoneal macrophages and Kupffer cells (Parwaresch and Wacker, 1984; Wacker et al., 1986); indeed, adoptively transferred Gr1^{lo} monocytes were shown to contribute to lung macrophage replacement after depletion (Landsman et al., 2007).

However, many observations conflict with the supposed monocyte origin of tissue-resident macrophages. For example, the first macrophages, called primitive macrophages, appear embryonically prior to the development of monocytes (Takahashi et al., 1989). In addition, monocytopenic animals have normal tissue macrophage density (Kuziel et al., 1997; Takahashi, 2001). Moreover, the cytokine interleukin-4 (IL-4) has recently been shown to promote local expansion of pleural-tissue macrophages in response to parasitic infection (Jenkins et al., 2011). We have demonstrated that central nervous system (CNS)-resident macrophages derive from yolk-sac macrophages and are maintained independently of monocytes (Ginhoux et al., 2010), which is in line with a recent report that a minor fraction of tissue-resident macrophages also derive from yolk-sac macrophages and are maintained independently of monocytes in the steady state (Schulz et al., 2012).

In this study, we sought to re-examine this controversy in the literature. Using a combination of monocyte fate mapping, parabiosis, depletion, radiation chimera, and adoptive transfer strategies, we show that, in contrast to the current dogma, tissue-resident macrophages repopulate locally throughout adult life, both in the steady state and after cell turnover, in a fashion that is predominantly independent of circulating monocytes.

RESULTS

Fate-Mapping Studies Indicate a Lack of Monocyte Contribution to Tissue-Resident Macrophages

To study the independence of tissue macrophages from circulating monocytes, we probed microarray data from the Immunological Genome Project to find fate-mapping candidate genes with which to trace monocyte progeny. Candidate genes were selected if they were absent in BM progenitors, were expressed in Gr-1^{hi} and Gr-1^{lo} monocytes, and were absent in certain tissue-resident macrophages. Monocytes and tissue macrophages were defined on the basis of their localization and the phenotypic markers described in Figure S1. We found two such candidates in *Mx1* and *S100a4* (Figures S1F and S1G). To map the fate of monocyte progeny, we crossed *Mx1-cre* (Kühn et al., 1995) and *S100a4-cre* (Bhowmick et al., 2004) mice with Rosa26^{Tomato} reporter animals (Madisen et al., 2010). In *Mx1-cre* × R26^{Tomato} animals, there was an increase of tdTomato signal in successive stages of myeloid differentiation and high expression in monocytes (39.7 ± 2.5% in Gr1^{hi} and 85.0 ± 2.4% in Gr1^{lo}) (Figures 1A and 1B; see also Figure S1I in the Supplemental Information available with this article online). Although a relatively high tdTomato signal can lead to multiple nondefinitive interpretations (as for peritoneal, red pulp, and BM macrophages in this model), the low number of tdTomato-positive macrophages (1.2 ± 0.35%) clearly indicate minimal contribution of monocytes to lung macrophages (Figures 1B, 1C, and S1J).

In the *S100a4-cre* × R26^{Tomato} fate-mapping model, we observed unexpectedly high expression of tdTomato in hematopoietic stem cells (HSCs) and other progenitors, despite the negligible expression of *S100a4* transcripts in these populations, suggesting *cre* recombination in more primitive hematopoietic cells that had given rise to the adult HSCs early in development (Figures 1D and S1G). Consistently, we also observed that more than 99% of monocytes expressed tdTomato (Figures 1E and S1J). However, macrophages from the lung, splenic red pulp, peritoneum, BM, and microglia (Figures 1F and S1J) had substantially lower tdTomato than did either hematopoietic progenitors or monocytes (Figures 1D and 1E), suggesting that tissue-resident macrophages originate independently not only of monocytes but also of adult hematopoietic progenitors.

We confirmed this possibility by using *Flt3-cre* × R26$^{Tomato/GFP}$ mice, which constitutively express tdTomato in all cells except the progeny of adult hematopoietic progenitors, which expressed GFP as a result of *Flt3-cre*-mediated recombination (Boyer et al., 2011). Although 90% of hematopoietic cells, circulating leukocytes, and lung and spleen monocytes expressed GFP (Figures 1G and S1K), GFP expression was very low in tissue macrophages (Figures 1H and S1K). This could not have been due to tissue-macrophage-specific shutdown of the Rosa26 locus because all the GFP^{-} tissue macrophages in the *Flt3-cre* × R26$^{Tomato/GFP}$ mice were tdTomato^{+}, indicating unabated activity of the Rosa26 locus (Figure S1K). Altogether, these three fate-mapping models strongly suggest that tissue macrophages develop independently of significant contribution of both blood monocytes and adult hematopoietic progenitors, consistent with recent findings (Schulz et al., 2012).

Parabiosis Indicates a Lack of Monocyte Contribution to Tissue-Resident Macrophages

To further establish the relationship between monocytes and tissue macrophages, we parabiotically joined CD45.2^{+} and congenic CD45.1^{+} mice and calculated the percentages of nonhost-derived cells. After 2 or 5 months of parabiosis, circulating Gr1^{hi} monocytes showed ~15% nonhost chimerism, whereas Gr1^{lo} monocytes had ~40% nonhost chimerism (Figure 2A and 2B), consistent with prior work (Jakubzick et al., 2008). If either monocyte population were differentiating into tissue macrophages in the steady state, nonhost chimerism would be expected to occur for tissue macrophages (as gated in Figures S1A–S1E) at a level comparable to that of monocyte chimerism. Consistent with their local self-maintenance and their derivation from embryonic macrophages—and not from monocytes (Ajami et al., 2007; Ginhoux et al., 2010)—microglia demonstrated <1% nonhost chimerism. However, we also found that lung, peritoneal, splenic, red-pulp, and BM macrophages also

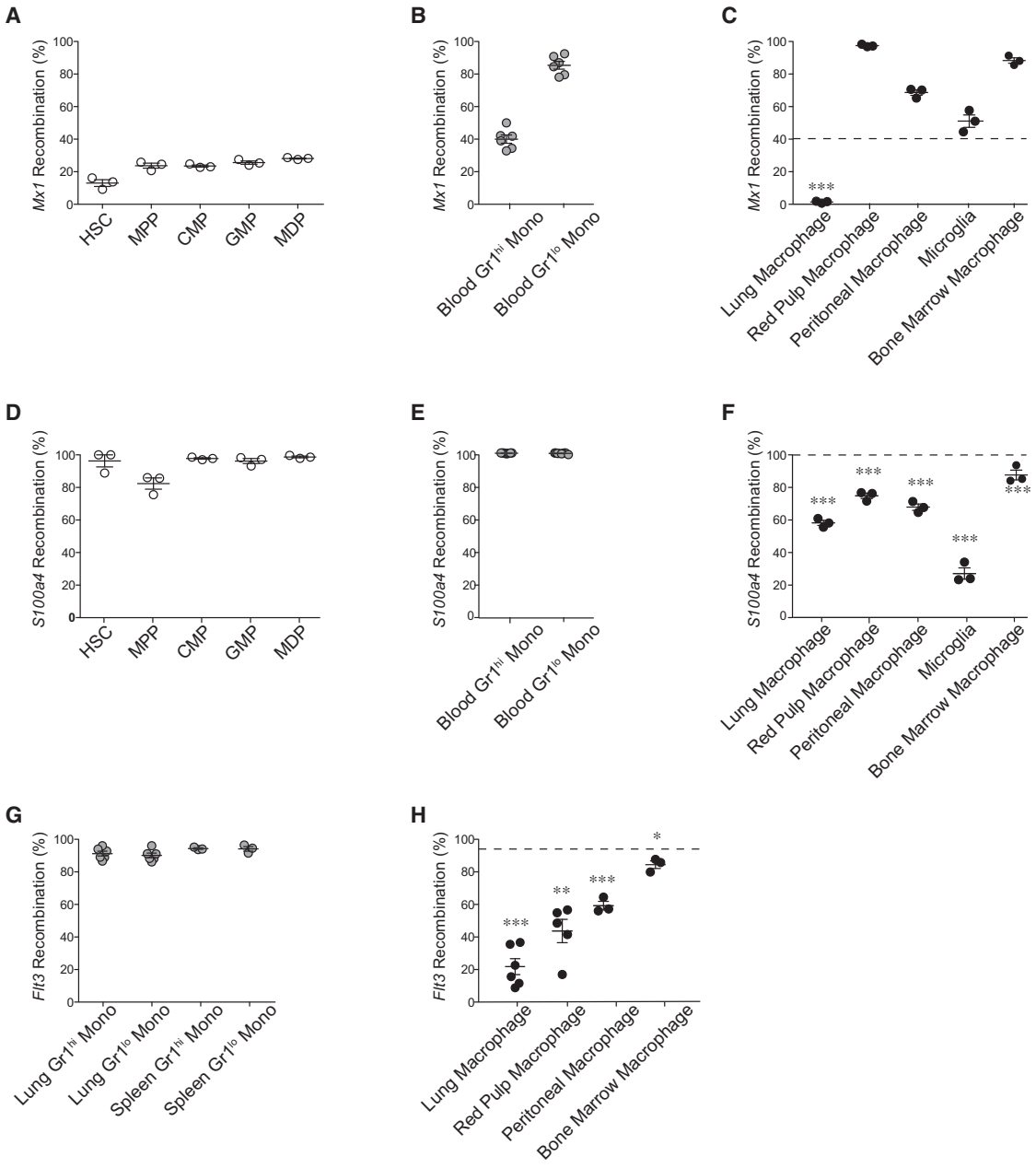

Figure 1. Fate Mapping Reveals that Tissue-Resident Macrophages Are Maintained Independently of Adult Hematopoiesis

(A–F) The percentage of tdTomato$^+$ cells among purified hematopoietic progenitors (A and D, open circles), monocytes (B and E, gray circles), and tissue-resident macrophages (C and F, black filled circles) in *Mx1-cre* × R26^{Tomato} (A and C) and *S100a4-cre* × R26^{Tomato} (D–F) are shown. Representative data from two separate experiments are shown.

(G and H) The percentage of GFP$^+$ cells among monocytes (G, gray circles) and tissue-resident macrophages (H, black circles) in *Flt3-cre* × R26$^{Tomato/GFP}$ mice are shown. Data were pooled from two independent experiments. Dashed lines in (C), (F), and (H) indicate the percent recombination in blood (C and F) or spleen (H) Gr1^{hi} monocytes. Abbreviations are as follows: LT-HSC, long-term hematopoietic stem cells; MPP, multipotent hematopoietic progenitors; CMP, common myeloid progenitors; GMP, granulocyte macrophage progenitor; and MDP, macrophage dendritic cell progenitor. Statistical significance is indicated as *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ in comparison to Gr1^{hi} monocytes. See also Figure S1.

demonstrated negligible chimerism after 2 and 5 months of parabiosis (Figures 2A and 2B).

To further accentuate the discordance between monocyte and tissue macrophage chimerism, we generated parabiotic mice between wild-type CD45.1$^+$ and either *Ccr2*$^{+/-}$ or *Ccr2*$^{-/-}$ CD45.2$^+$ mice. Although *Ccr2*$^{+/-}$CD45.2$^+$ parabionts showed nonhost chimerism similar to that of wild-type parabionts (compare Figures 2A and 2B with Figure S2A), *Ccr2*$^{-/-}$CD45.2$^+$ parabionts demonstrated ~70% and ~90% nonhost chimerism among Gr1^{hi} and Gr1^{lo} monocytes, respectively (Figure 2C)

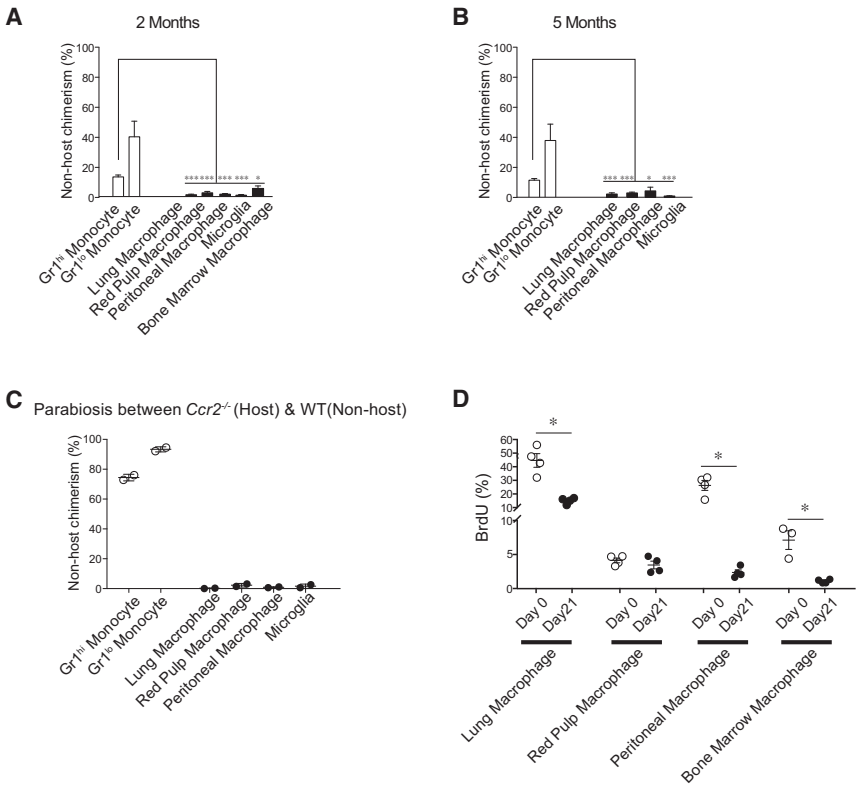

Figure 2. Parabiosis Studies Indicate that Tissue-Resident Macrophages Are Maintained Independently of Monocytes

(A and B) C57BL/6 CD45.2$^+$ and CD45.1$^+$ parabionts were surgically connected for 2 months (A) or 5 months (B). Bars show the percentage nonhost cells among peripheral blood monocytes (white bars) and tissue-resident macrophages (black bars) in host parabionts (n = 4–6). Representative data from two separate experiments are shown as means +/− SEM. Statistical significance is indicated by *p < 0.05, **p < 0.01, and ***p < 0.001 in comparison to Gr1^{hi} monocytes.

(C) Parabiotic pairs were generated from wild-type CD45.1$^+$ mice and *Ccr2*$^{-/-}$ CD45.2$^+$ mice. The percentage of nonhost cells among peripheral blood monocytes (open circles) and tissue macrophages (filled circles) in the CD45.2$^+$ mouse was analyzed 2 months after surgery.

(D) C57BL/6 mice were pulsed with BrdU for 3 weeks (1 mg intraperitoneally daily), and BrdU incorporation in tissue macrophages was assessed 1 day (day 0, open circles) and 22 days (day 21, filled circles) after the last pulse. An asterisk indicates p < 0.05. See also Figure S2.

because of defective monocyte emigration from the BM into the circulation of *Ccr2*$^{-/-}$ animals (Serbina and Pamer, 2006). Nonetheless, nonhost chimerism of lung, red-pulp, peritoneal, and BM macrophages remained negligible after 2 months of parabiosis (Figure 2C). Importantly, this discordance in monocyte versus macrophage chimerism was still observed after 7 months and after 1 year of parabiosis (Figure S2B). To assess whether the absence of monocyte contribution to macrophage homeostasis could be due to their prolonged half-life in tissues, we used bromodeoxyuridine (BrdU) labeling to measure macrophage turnover in situ. We observed substantial turnover of tissue-resident macrophages over the course of 21 days with the exception of spleen red-pulp macrophages (Figure 2D), suggesting that the lack of nonhost chimerism up to one year after parabiosis does not result from a long lifespan of tissue macrophages or a lack of opportunity for monocyte replacement.

Tissue-Resident Macrophages Repopulate through Local Proliferation after Nongenotoxic Ablation

The preceding data suggest that tissue macrophages locally self-maintain in the steady state with minimal contribution from monocytes. We next asked whether this paradigm persisted after enforced cell turnover. To this end, we utilized CD169-DTR animals, which express the diphtheria toxin receptor (DTR) downstream of the Siglec1 (also called CD169 or Sialoadhesin) locus (Miyake et al., 2007). Administration of diphtheria toxin (DT) into CD169-DTR animals allows for specific depletion of certain tissue-resident macrophages without ablating monocytes or dendritic cells (Asano et al., 2011; Chow et al., 2011; Miyake et al., 2007). Short-term DT administration into CD169-DTR animals resulted in efficient reduction of BM and lung macrophages (Figure 3A); however, peritoneal macrophages and red-pulp macrophages were not reduced (data not shown), precluding analyses of these macrophages in the CD169-DTR depletion model.

We generated CD169$^{DTR/+}$*Ccr2*$^{-/-}$ mice to determine whether CCR2-dependent migration of candidate progenitor cells, including monocytes and hematopoietic progenitors, contribute to the recovery of tissue-resident macrophages after turnover (Boring et al., 1997). Consistent with prior work (Kuziel et al., 1997; Serbina and Pamer, 2006), *Ccr2*$^{-/-}$ mice indeed had lower circulating monocyte numbers (Figure S3A) and also lower steady-state monocyte numbers in the lung and spleen (Figure S3B and data not shown) than did *Ccr2*$^{+/-}$ littermates, but they had comparable numbers of BM and lung macrophages (Figures 3B and 3C). Consistent with the lack of a CCR2-dependent contribution to steady-state tissue macrophages, we also found that both the extent of the depletion and the kinetics of the recovery were equivalent in DT-infused CD169$^{DTR/+}$*Ccr2*$^{+/-}$ and CD169$^{DTR+/-}$ *Ccr2*$^{-/-}$ mice (Figures 3B and 3C), indicating that the recovery of the tissue-resident-macrophage compartment is independent of CCR2-dependent cells such as monocytes (Serbina and Pamer, 2006) and hematopoietic progenitors (Si et al., 2010).

To further examine the contribution of circulating hematopoietic cells to the recovery of tissue-resident macrophages after nongenotoxic ablation, we infused 7.5 × 10^7 BM cells isolated from CD45.1$^+$ congenic mice into CD169$^{DTR/+}$ CD45.2$^+$ recipient mice without performing prior conditioning. With this adoptive transfer approach, we obtained a low but stable engraftment of donor BM progenitors and, consequently, stable donor monocyte chimerism in the recipient blood (4.4 ± 1.5% for Gr1^{hi} monocytes and 7.2 ± 1.6% for Gr1^{lo} monocytes) (Figure 3D). Consistent with

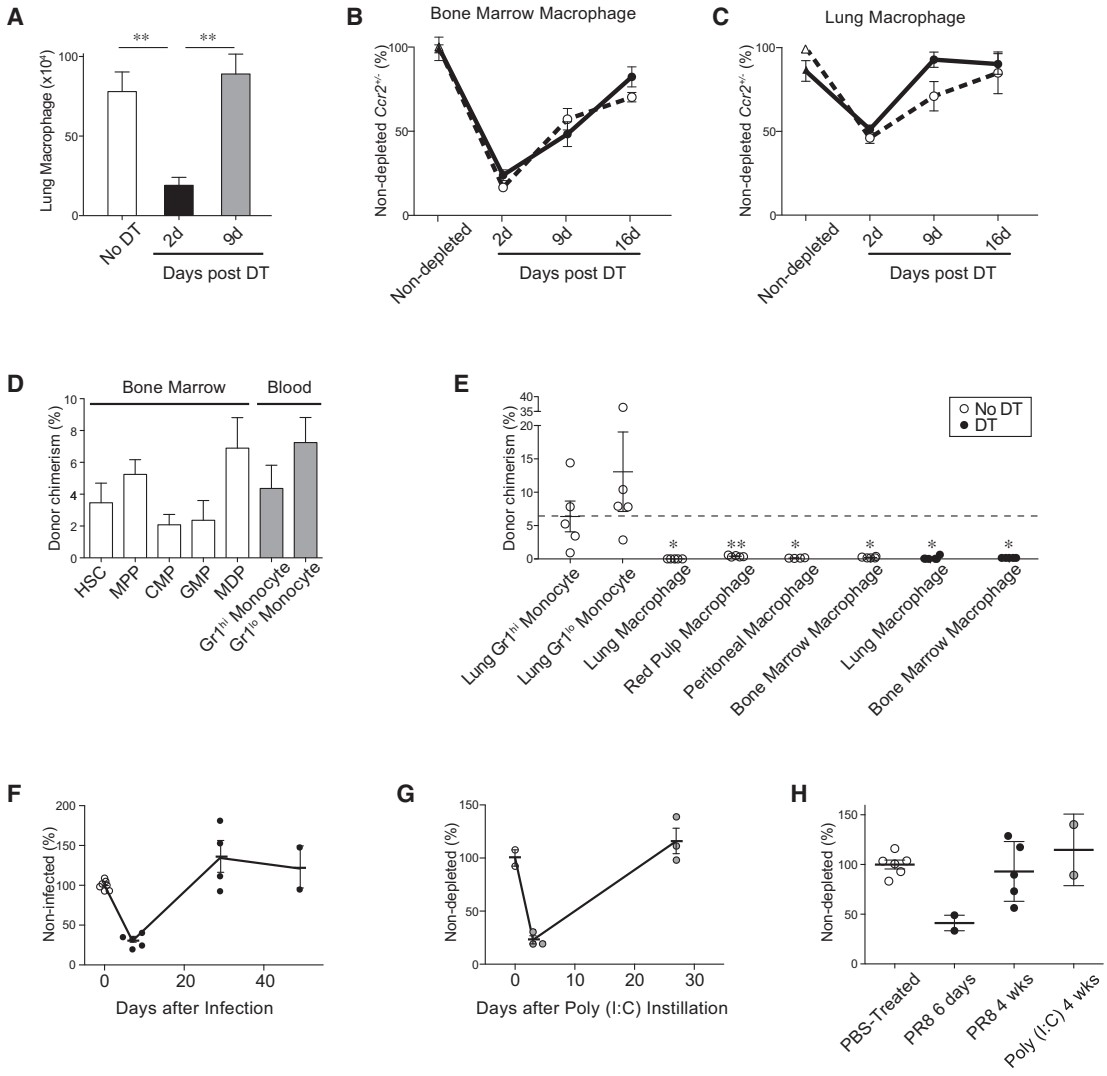

Figure 3. Tissue-Resident Macrophages Repopulate Locally Independently of CCR2$^+$ Progenitors

(A) CD169$^{DTR/+}$ mice were injected intraperitoneally with 10 μg/kg DT (n = 5, filled bars) or control diluent (n = 3, open bars) on days 1 and 4, and the absolute numbers of lung macrophages were enumerated 2 days and 9 days after the last injection. Representative data from three separate experiments are shown as means +/– SEM. A double asterisk indicates $p < 0.01$.

(B and C) CD169$^{DTR/+}$ × *Ccr2*$^{+/-}$ (open circles, n = 5/time point) and CD169$^{DTR/+}$ × *Ccr2*$^{-/-}$ mice (filled circles, n = 6/time point) were injected with DT on day 0, and the numbers of BM macrophages (B) and lung macrophages (C) were analyzed at 2, 9, and 16 days after DT administration. CD169$^{+/+}$ × *Ccr2*$^{+/-}$ (open triangle) and CD169$^{+/+}$ × *Ccr2*$^{-/-}$ (filled triangle) mice treated with DT were used as nondepleted controls. The absolute cell numbers are represented as relative values for which the mean absolute number of lung macrophages in nondepleted CD169$^{+/+}$ × *Ccr2*$^{+/-}$ mice was set at 100%. Data were pooled from two independent experiments and are shown as means +/– SEM.

(D and E) CD169$^{DTR/+}$ (CD45.2$^+$, n = 5) mice were infused with 7.5 × 10^7 congenic CD45.1$^+$ BM cells, and DT (filled circles) or PBS (open circles) administration followed on days 4 and 7 after BM cell transfer. The percentages of donor-derived CD45.1$^+$ cells among (D) BM progenitor cells (white bars) and blood monocytes (gray bars) and (E) lung monocytes and tissue macrophages were analyzed sixteen days after the transfer. Representative data from three independent experiments are shown as means +/– SEM. Statistical significance is indicated by *$p < 0.05$ and **$p < 0.01$ in comparison to Gr1^{hi} monocytes.

(F–H) *S100a4-cre* × R26^{Tomato} mice were infected intranasally with 100 pfu PR8 influenza virus (F and H, filled circles) or instilled intranasally with 50 μg Poly (I:C) (G and H gray circles) or control PBS (F–H, open circles). The numbers of lung macrophages at different times after PR8 infection (F) or Poly (I:C) injection (G) and numbers of tdTomato$^-$ lung macrophages at 6 days and 4 weeks following PR8 infection or Poly (I:C) injection are shown (H). The absolute cell numbers are represented as relative values for which the mean absolute number of lung macrophages in noninfected or non-Poly(I:C)-instilled control animals was set at 100%. Data are represented as means +/– SEM. See also Figure S3.

the parabiosis studies (Figure 2), we observed that the percentage of CD45.1$^+$ donor-derived cells among lung, red-pulp, peritoneal, and BM macrophages in recipient mice without DT injection was negligible (0.002% ± 0.002%, 0.34% ± 0.06%, 0.05% ± 0.02%, and 0.07% ± 0.05%, respectively) (Figure 3E). The depletion of tissue-resident macrophages and their subsequent recovery 9 days later did not increase the donor-tissue macrophage chimerism (Figure 3E), indicating that circulating hematopoietic progenitors or monocytes did not contribute to the recovery of the tissue-resident macrophages.

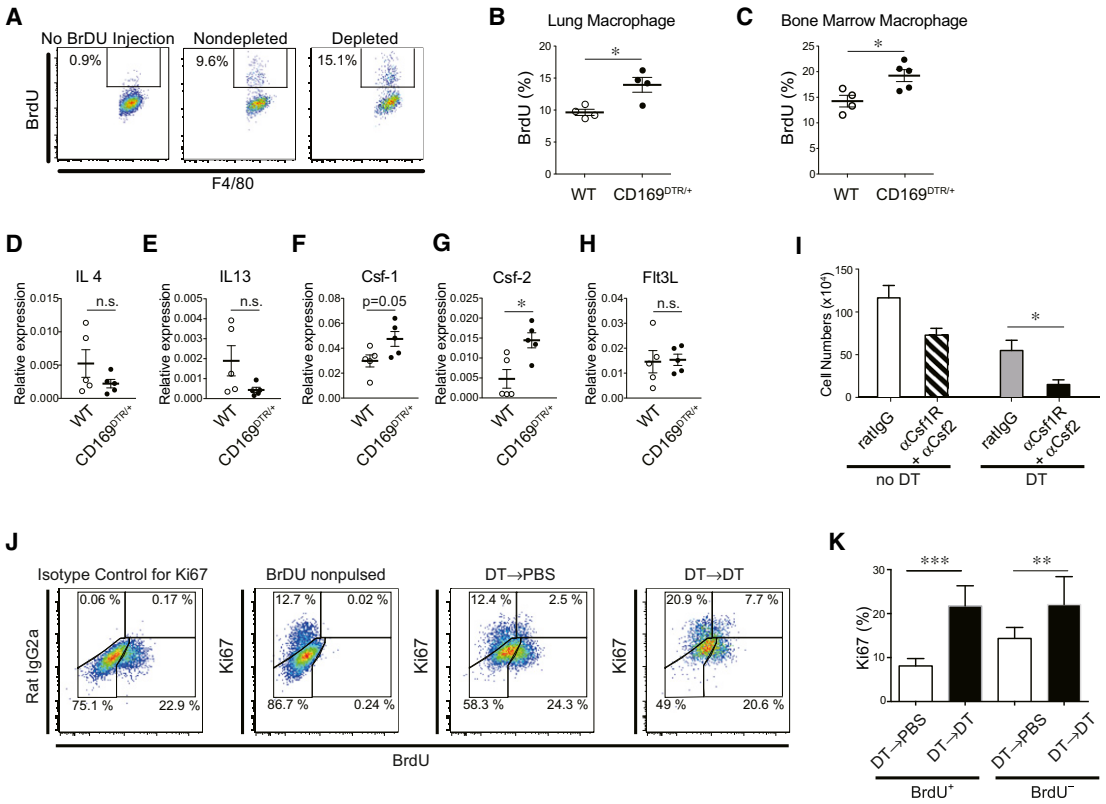

Figure 4. Repopulation of Lung-Tissue-Resident Macrophages Is Dependent on Local Cytokine Production

(A–H) Wild-type or CD169$^{DTR/+}$ mice were administered 10 μg/kg DT on days −5 and −2 and injected intraperitoneally with 1 mg BrdU/mouse on day −1 prior to analysis. (A) Flow-cytometry plots depict the percentage of BrdU-positive cells among total lung macrophages in non-BrdU-injected mice (left panel), WT mice treated with DT (center panel), or CD169$^{DTR/+}$ mice treated with DT (right panel). (B and C) Quantitation of the percentage of BrdU^{+} cells among lung macrophages (B) and BM macrophages (C) in WT (open circles) and CD169$^{DTR/+}$ (filled circles) mice treated with DT. (D–H) mRNA was purified from lung homogenates and expression of IL-4 (D), IL-13 (E), CSF-1 (F), CSF-2 (G), and Flt3L (H) were quantified by real-time PCR relative to the expression of Gapdh. (I) CD169$^{DTR/+}$ mice were injected with PBS or DT on days −12 and −9 prior to analysis followed by i.n. injection of 600 μg of anti-Csf-1R antibody (clone: AFS98) on days −8, −6, −4, and −1 and 100 μg of anti-Csf-2 neutralizing antibody (clone: MP122E9) on days −7, −5, −2 prior to the analysis. The numbers of lung-tissue-resident macrophages in mice that received no Dt and either rat IgG (open bar, n = 16) or both anti-Csf-1R mAb and anti-Csf-2 mAb (hatched bar, n = 5) and in mice that received DT and either control rat IgG (gray filled bar, n = 9) or both anti-Csf-1R mAb and anti-Csf-2 mAb (black filled bar, n = 10). Data were pooled from three independent experiments and are shown as means +/– SEM. (J and K) CD169$^{DTR/+}$ mice were treated with 10 μg/kg DT on day −16, pulsed with 1 mg/day BrdU on days −15 through −5, and reinjected with DT (black bars, n = 8) or PBS (white bars, n = 8) on day −2 prior to analysis. Mice were sacrificed on day 0, and BrdU incorporation and Ki67 expression were analyzed by flow cytometry. Representative FACS plots of Ki67 and BrdU staining in lung macrophages (J) are shown. Ki67 expression (cycling cells) among BrdU^{+} fractions and BrdU^{-} fractions of lung macrophages were quantified (K). Data were pooled from two independent experiments and are shown as means +/– SEM. Statistical significance is indicated by *p < 0.05, **p < 0.01, and ***p < 0.001. See also Figure S4. αCsf1R and αCsf2 indicate anti-Csf1R and anti-Csf2, respectively.

The recent observation that macrophages are depleted after Toxoplasma infection led us to examine the contribution of monocytes to tissue-resident macrophages in an infectious setting (Goldszmid et al., 2012). We intranasally infected *S100a4-cre* × R26^{Tomato} mice with the PR8 strain of the influenza virus, which resulted in dramatic lung-macrophage cytoablation 6 days after infection, substantial infiltration of Gr1^{hi} monocytes, and recovery of macrophage numbers by 4 weeks after infection (Figures 3F, S3C, and S3D). Consistent with Figure 1, 35%–40% of lung macrophages were tdTomato^{-}. If adult hematopoietic progenitors or monocytes, which are >99% tdTomato^{+}, were replacing lung macrophages, one would expect that the absolute number of tdTomato^{-} macrophages would not recover after influenza infection and would instead be replaced by tdTomato^{+} progenitors. On the contrary, we observed that the number of tdTomato^{-} macrophages returned to uninfected baseline numbers 4 weeks after infection, indicating recovery from local repopulation of tdTomato^{-} macrophages (Figure 3H). Similarly, when we intranasally instilled *S100a4-cre* × R26^{Tomato} mice with 50 μg of Poly (I:C) to induce macrophage cytoablation and monocyte infiltration (Figures 3G, S3E, and S3F), tdTomato^{-} macrophages returned to normal numbers 4 weeks after instillation (Figure 3H).

Because we observed no replacement of tissue-resident macrophages by circulating progenitor candidates, we hypothesized that the replenishment of tissue-resident macrophages occurred via local proliferation. Assessing proliferation with a short pulse of BrdU, we observed that macrophage depletion in CD169-DTR mice resulted in a significant increase in the proliferation of lung and BM macrophages (Figures 4A–4C). In CD11c-DTR

transgenic mice, DT administration depleted red-pulp but not lung macrophages (Figure S4A), as previously reported (Meredith et al., 2012). Accordingly, we found increased macrophage proliferation in the red pulp but not in lung tissues (Figure S4B). Using systemic administration of clodronate liposomes, we also observed that a depletion of lung, red-pulp, and BM macrophages was associated with enhanced subsequent local proliferation (Figures S4C–S4E). Obtained from three distinct conditional depletion models, our results collectively reveal that macrophages enhance proliferation in order to repopulate locally after cell turnover.

Although IL4 has been shown to be the critical cytokine that promotes local expansion of the pool of tissue-resident macrophages in parasitic infection (Jenkins et al., 2011), the expression of IL4 and IL13, which also utilizes the IL4 receptor, did not increase in the lungs of macrophage-depleted mice (Figures 4D and 4E). On the other hand, we observed an increase in the expression of Colony-stimulating factor 1 (Csf-1; also called M-Csf) and Csf-2 (also called GM-Csf), but not fms-like tyrosine kinase 3 ligand (Flt3L) in the lung tissue of macrophage-depleted mice (Figures 4F–4H). To determine whether recovery of the lung-macrophage compartment after ablation was dependent on Csf-1 and Csf-2 cytokines, we administered anti-Csf-1R and anti-Csf-2 blocking antibodies after macrophage depletion. The recovery of tissue-resident macrophages was impaired in the cytokine-blocked mice compared to rat IgG-treated control mice (Figure 4I). Anti-Csf-1R and anti-Csf-2 blocking antibodies also reduced tissue macrophages in nondepleted mice. These data indicate that local repopulation of macrophages in both the steady state and upon enforced depletion is dependent on Csf-1R and/or Csf-2 signaling.

Next, we examined whether the ability of macrophages to repopulate was restricted to a particular progenitor population or whether all macrophages could self-renew in a stochastic manner. Thus, we depleted lung macrophages in the CD169-DTR model and subsequently delivered daily pulses of BrdU injections for 10 days to label proliferating cells. Two days after completion of the day 10 pulse, the CD169-DTR mice were injected with DT once again. Two days later, mice were sacrificed, and we used an antibody against the cell-cycle protein Ki-67 to distinguish between cycling macrophages that had proliferated after the initial DT injection (Ki-67$^+$BrdU$^+$) and the newly proliferating population (Ki-67$^+$BrdU$^-$). Ki-67 was equally increased among BrdU$^+$ and BrdU$^-$ macrophages, indicating that repopulation capacity was a stochastic event and not restricted to a subset of macrophages. (Figures 4J and 4K). In contrast, DT injection failed to induce B cell proliferation, establishing that induction of cellular proliferation upon DT injection in CD169-DTR mice was specific to macrophages (Figures S4F and S4G). Altogether, these data suggest that differentiated macrophages proliferate locally and stochastically during cell turnover.

Recovery of Tissue-Resident Macrophages after Genotoxic Insult Occurs via Donor Circulating Progenitors

With the exception of microglia (Ginhoux et al., 2010) (Figure 5A), most tissue-resident macrophages derive from donor bone marrow hematopoietic precursers after lethal irradiation and bone marrow transplantation (Haniffa et al., 2009; Virolainen, 1968), indicating that unlike in the CD169-DTR depletion and inflammatory models above, lethal irradiation represents an insult that facilitates impairment of local repopulation capacity and thus donor engraftment. In this context, in which macrophages do arise from donor cells, we sought to determine whether the kinetics of tissue-resident-macrophage recovery was consistent with derivation from monocytes. We found that within 2 weeks of bone marrow transplantation, all Gr1^{hi} and Gr1^{lo} monocytes in the peripheral blood, lung, and spleen were of donor origin (Figures 5A–5C). Although tissue-resident macrophages in the lung, splenic red pulp, peritoneum, and BM were eventually replaced by donor cells (Figures 5B–5D), donor repopulation of macrophages was markedly delayed in comparison to that of monocytes, suggesting that monocytes might not contribute to the recovery of tissue-resident macrophages after genotoxic insult.

Host Tissue-Resident Macrophages Retain the Capability to Self-Maintain Even after Genotoxic Insult

The inability of host tissue-resident macrophages to regenerate chimerism after bone marrow transplantation could potentially be due to irreversible radiation-induced destruction of tissue-resident macrophages or incomplete radiation-induced suppression of tissue-resident-macrophage self-renewal. In order to distinguish between these two possibilities, we evaluated a system in which donor repopulation of the tissue-resident-macrophage compartment was specifically impaired. To achieve this, we infused 1 × 10^6 wild-type or *Csf1r*$^{-/-}$ fetal liver cells into lethally irradiated adult animals to establish a transplantation system in which donor-derived cells were deficient in Csf-1R. Four months after transplantation, a time point at which monocytes were completely of donor origin both in [WT → WT] chimera and [*Csf-1r*$^{-/-}$ → WT] chimeras (Figure S5A), we enumerated donor-derived and host-derived tissue-resident macrophages. In the lungs of the recipients of *Csf-1r*$^{-/-}$ fetal liver, there was a slight nonsignificant reduction in the number of donor-derived tissue-resident macrophages when these mice were compared to recipients of WT fetal liver cells (Figures 5E and S5B). Nonetheless, this slight reduction in donor-derived macrophages was associated with a substantial expansion of the host macrophages (Figure 5E), suggesting that host tissue-resident macrophages retained the capacity to self-renew in the context of reduced competition from donor-derived cells. This impaired donor contribution and consequent repopulation of host tissue-resident macrophages was even more profound in the spleen and BM; the entire deficit in donor-derived splenic red-pulp and BM macrophages was recovered by the expansion of the host tissue-resident-macrophage compartment (Figures 5F, 5H, S5C, and S5E). In the peritoneum, there was also a substantial reduction in donor-derived macrophages in recipients of *Csf1r*$^{-/-}$ fetal liver cells, and there was a small, although not statistically significant, expansion of host peritoneal macrophages (Figures 5G and S5D). Together, these results show that the recovery of the tissue-resident macrophage compartment in the spleen, peritoneum, and BM is dependent on Csf-1R signaling, whereas it is partially dependent on Csf-1R in the lung. Furthermore, at least in the lung, spleen, and BM, host tissue-resident macrophages can expand in the setting of reduced donor competition.

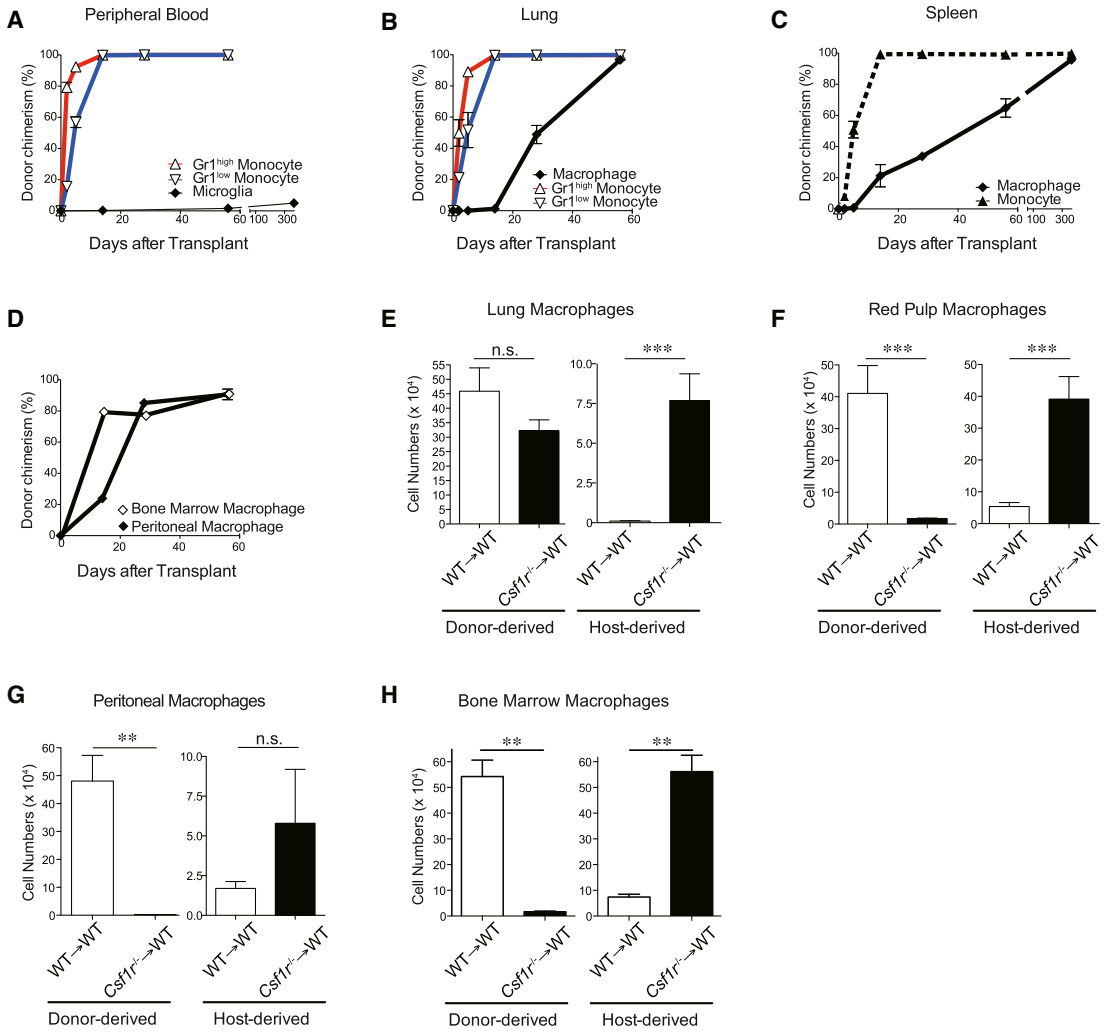

Figure 5. Ionizing Irradiation Does Not Abrogate Host Tissue Macrophage Repopulation Potential

(A–D) C57BL/6 CD45.2$^+$ mice were lethally irradiated and transplanted with 5 × 10^6 congenic BM CD45.1$^+$ cells. The percentage of donor (CD45.1$^+$) cells among circulating monocytes and CNS microglia (A); monocytes and/or macrophages in the lung (B); spleen (C); BM (D); and peritoneum (D) are shown (n = 3–4/time point). (E–H) C57BL/6 CD45.1$^+$ mice were lethally irradiated and transplanted with 1 × 10^6 fetal liver cells isolated from C57BL/6 CD45.2$^+$ *Csf-1r*$^{-/-}$ (black bars, n = 7) mice or control littermates (white bars, n = 7). The absolute numbers of host and donor tissue-resident macrophages in the lung (E), spleen (F), peritoneum (G), and BM (H) were enumerated 4 months after transplantation. Data were pooled from two independent experiments and are shown as means +/– SEM. Statistical significance is indicated by **p < 0.01 and ***p < 0.001. See also Figure S5.

Because Csf-2 is critical to lung-macrophage homeostasis (Trapnell and Whitsett, 2002) (Figures S6A and S6B), we sought to examine whether impaired Csf-2-dependent donor repopulation could also result in the development of host lung-macrophage expansion after lethal irradiation. Two months after bone marrow transplantation of wild-type or Csf-2-receptor-deficient (*Csf-2r*$^{-/-}$) CD45.2$^+$ cells into lethally irradiated CD45.1$^+$ mice, all circulating monocytes were completely donor derived (Figure S6C). Although recipients of wild-type BM achieved donor-derived recovery of the lung-macrophage compartment, we observed that donor-derived recovery of lung macrophages was substantially reduced in *Csf-2r*$^{-/-}$ BM chimeric mice (Figures 6A and S6D). Importantly, the severe impairment of donor-derived-macrophage development resulted in a compensatory expansion in the pool of host tissue-resident macrophages over time (Figures 6A and S6E). Mice deficient in Csf-2 (Stanley et al., 1994) and Csf-2R (Robb et al., 1995) manifest a severe lung inflammatory disease characteristic of clinical alveolar proteinosis as a result of impaired surfactant clearance by defective pulmonary macrophages. Defects in Csf-2 and Csf-2R (Trapnell et al., 2003) have been described in patients with this disorder. We evaluated whether the host macrophages that recovered from genotoxic insult were functionally competent by examining their ability to clear surfactant and prevent the development of alveolar proteinosis in these chimeras. In comparison to wild-type mice, naive *Csf-2r*$^{-/-}$ mice had significantly elevated alveolar protein concentration (Figure 6B). Furthermore, the lung histology showed conspicuous granular eosinophilic material within the alveolar spaces, as visualized with H&E staining, and this material was positive for periodic

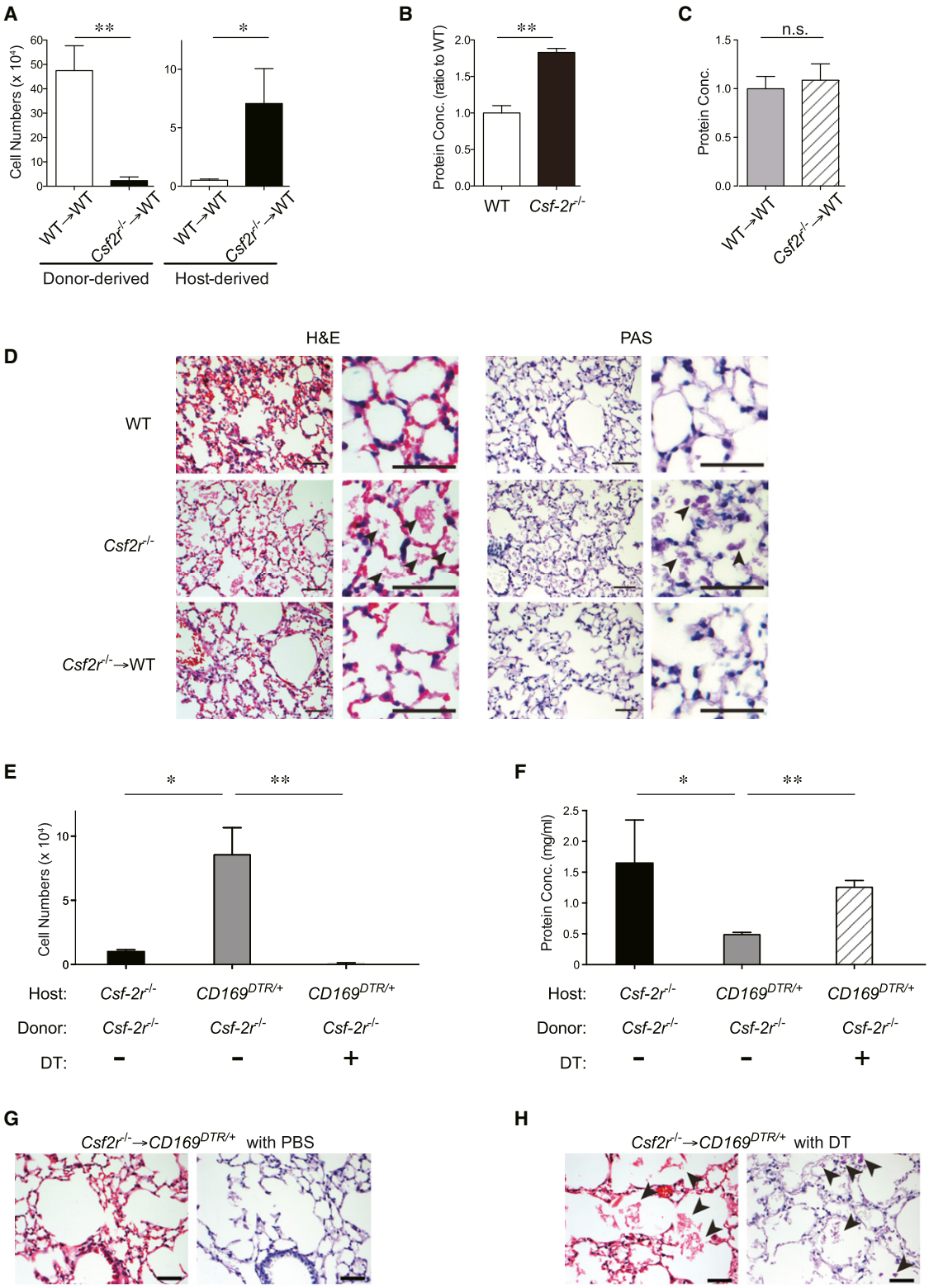

Figure 6. Lung Macrophages Repopulate Locally after Lethal Irradiation and Promote Lung-Tissue Integrity

(A–D) CD45.1$^+$ mice were lethally irradiated and transplanted with 5 × 10^6 BM cells isolated from CD45.2$^+$ *Csf-2r*$^{-/-}$ (black bars, n = 6) mice or control CD45.2$^+$ littermates (white bars, n = 5). (A) The absolute numbers of donor- and host-derived lung macrophages in right lung lobes were enumerated 2 months after bone marrow transplantation. (B and C) Protein concentrations in the bronchial alveolar lavage fluid (BALF) were quantified in wild-type mice (white bar), *Csf-2r*$^{-/-}$ mice (black bar), and wild-type mice transplanted with wild-type (gray bar) or *Csf-2r*$^{-/-}$ (hatched bar) BM (n = 3/group) 2 months after bone marrow transplantation. Data

(legend continued on next page)

acid-shiff (PAS), indicative of clinical alveolar proteinosis (Figure 6D). In contrast, [*Csf-2r*$^{-/-}$→WT] chimeras had an alveolar protein concentration comparable to that of [WT→WT] chimeras (Figure 6C). Blinded examination by two independent lung pathological examiners did not detect any pathology in either [*Csf-2r*$^{-/-}$→WT] or [WT→WT] chimeras (Figures 6D and S6F). To further assess the functional ability of self-renewing host macrophages to clear alveolar proteins, we reconstituted CD169$^{DTR/+}$ mice were with *Csf-2r*$^{-/-}$ BM cells. We injected BM chimeric mice twice weekly with either DT or PBS for up to 8 weeks after transplant to deplete host CD169^{+} lung macrophages. We found that the inability of host macrophages to repopulate in DT-treated [*Csf-2r*$^{-/-}$→CD169$^{DTR/+}$] chimeric mice was associated with elevated bronchoalveolar lavage fluid (BALF) protein concentrations and the development of alveolar proteinosis (Figures 6E, 6F, and 6H), whereas PBS-treated [*Csf-2r*$^{-/-}$→CD169$^{DTR/+}$] chimeric mice were protected from lung disease (Figures 6E–6G). Hence, host-derived lung macrophages can resist radiation injury and functionally repopulate when donor repopulation is impaired, and this expansion of host-derived wild-type macrophages is sufficient to prevent the development of alveolar proteinosis.

DISCUSSION

Recent findings have called into question the dogma that monocytes constitutively give rise to tissue-resident macrophages (Ajami et al., 2007; Ginhoux et al., 2010; Jenkins et al., 2011; Schulz et al., 2012). In order to address this controversy, we took advantage of animal models that have allowed us to comprehensively re-examine the origin of tissue-resident macrophages. Utilizing three distinct fate-mapping models, parabiosis studies, and adoptive transfer of total BM cells, we used discordant chimerism between monocytes and tissue macrophages to demonstrate that monocytes are not the progenitors of lung, splenic red-pulp, peritoneal, or BM tissue macrophages in the steady state. Even when host tissue-resident macrophages were eliminated in the lung and BM and forced to turn over, repopulation of macrophages still occurred independently of circulating monocytes. Our finding that these tissue-resident macrophages can extensively self-maintain independently of adult HSCs in the steady state complements the recent report that the yolk-sac-derived fraction of splenic, hepatic, and pancreatic macrophages can also self-maintain (Schulz et al., 2012) and goes further in demonstrating that this capacity for self-maintenance is not restricted to the steady state but is preserved after cell turnover. These results show that, similar to Langerhans cells and microglia, several tissue macrophages self-renew in situ and can repopulate locally after tissue injuries (Ajami et al., 2007; Merad et al., 2002).

If circulating monocytes and hematopoietic progenitors are not contributing, then how are tissue-resident macrophages maintained in the steady state and after turnover? In line with previous reports of the proliferation of tissue-resident macrophages in the steady state(Daems and de Bakker, 1982; Jenkins et al., 2011), we found that nongenotoxic depletion of lung macrophages was followed by local proliferation and enhanced local cytokine production of Csf-1 and Csf-2. Consistent with the importance of these two cytokines for lung-macrophage repopulation after cell turnover, concomitant blockade of Csf-1R and Csf-2 signaling compromised lung-macrophage repopulation after nongenotoxic depletion. Thus, we conclude that tissue-resident macrophages are capable of local repopulation in the steady state and after cell turnover at least in some tissues. Local proliferation potential was stochastic and not restricted to a subset of tissue macrophages.

Importantly, we also found that host tissue-resident macrophages can repopulate locally after macrophage cytoablation caused by infection with influenza or instillation of Poly (I:C), which complements prior results showing that pleural tissue-resident macrophages can proliferate locally in mice in response to parasitic infection (Jenkins et al., 2011). Unfortunately, we were unable to trace the monocyte progeny in inflamed tissues, and it is unclear at this point whether monocyte-derived macrophages transiently infiltrate inflamed tissues or remain in the tissues once the inflammation resolves.

In humans, there have been numerous reports of congenital monocytopenia whose cause was not associated with a reduction in tissue-resident macrophages. In reticular dysgenesis, a rare inherited immunodeficiency characterized by the lack of blood monocytes, neutrophils, and lymphocytes, macrophages are present in normal numbers in the dermis and in the atrophic lymphoid tissues of affected individuals (Emile et al., 2000). Moreover, there was a report of 18 patients who had an autosomal-dominant susceptibility to opportunistic infections and monocytopenia and who had normal numbers of dermal macrophages (Vinh et al., 2010). In addition, in four patients with a syndrome involving monocytopenia and a deficiency of dendritic, B, and NK cells, dermal macrophages were found to be present (Bigley et al., 2011). Similarly, a patient with an IRF8-null mutation was recently found to have a block in monocyte and DC differentiation and increased susceptibility to mycobacterium. However, despite profound peripheral monocytopenia, macrophages were present in the lymph nodes and in the BM of an

were pooled from two independent experiments and are shown as means +/– SEM. (D) Representative sections of left lung lobes stained with H&E (left) and PAS (right) were obtained from steady-state wild-type (WT) mice (top), steady-state *Csf-2r*$^{-/-}$ mice (center), and WT mice transplanted with *Csf-2r*$^{-/-}$ (bottom) BM. Arrow heads indicate PAS^{+} eosinophilic material within the alveolar spaces. The scale bar represents 25 μm.

(E–H) *Csf-2r*$^{-/-}$ mice and CD169$^{DTR/+}$ mice were lethally irradiated and reconstituted with 5 × 10^{6} BM cells isolated from C57BL/6 WT or *Csf-2r*$^{-/-}$ mice. A group of CD169$^{DTR/+}$*Csf-2r*$^{+/+}$ recipients were injected with DT (10 μg/kg, twice weekly) starting from day +3 after transplantation. The absolute numbers of macrophages in the right lung lobes (E) and protein concentrations in BALF (F) in [*Csf-2r*$^{-/-}$ into *Csf-2r*$^{-/-}$] (black bars, n = 3) and [*Csf-2r*$^{-/-}$ into CD169$^{DTR/+}$] chimera treated with PBS (gray bars, n = 6) or DT (hatched bars, n = 6) were enumerated 2 months after transplantation. Data were pooled from two independent experiments and are shown as means +/– SEM. Paraffin sections of the left lung lobes of recipients of [*Csf-2r*$^{-/-}$ into CD169$^{DTR/+}$] chimeras treated with PBS (G) or DT (H) at 2 months after transplantation were stained with H&E (left panel) and PAS (right panel). Sections isolated from [*Csf-2r*$^{-/-}$ into CD169$^{DTR/+}$] chimera treated with DT (H) demonstrate granular eosinophilic material positive for PAS within the alveolar spaces (arrow heads), whereas sections from [*Csf-2r*$^{-/-}$ into CD169$^{DTR/+}$] chimera treated with diluent (G) demonstrate normal alveolar structure. Scale bars represent 25 μm. See also Figure S6. Statistical significance is indicated by *p < 0.05 and **p < 0.01.

IRF8-mutated patient (Hambleton et al., 2011). The normalcy of macrophages in these monocytopenic patients further suggests that tissue-resident macrophages can develop in the absence of monocytes in humans.

It has been known for decades that tissue-resident macrophages are replaced, albeit slowly, by BM-derived cells after bone marrow transplantation (Thomas et al., 1976; Virolainen, 1968), and in fact, these results were used as an early argument for a circulating contribution to tissue-resident macrophages. However, our data indicates that there is extensive recovery of host-derived tissue-resident macrophages when donor-derived repopulation is impaired, as in the chimeras generated from *Csf1r*$^{-/-}$ fetal liver or *Csf2r*$^{-/-}$ BM. These data are consistent with the observation that fractionation of a lethal irradiation dose allows substantial recovery of host-derived lung macrophages (Tarling et al., 1987). Importantly, this expansion of host-derived wild-type macrophages is functional; the macrophages prevented the development of alveolar proteinosis observed in transplant recipients of *Csf2r*$^{-/-}$ BM. These observations in the myeloablative setting dovetail nicely with our findings that tissue-resident macrophages self-maintain locally throughout adult life in the steady state and after nongenotoxic turnover of tissue-resident macrophages. Besides the well-described effect that Csf-2 has on macrophage phagocytosis (Trapnell et al., 2003), its control of macrophage numbers is probably another mechanism by which defects in Csf-2 and Csf-2R result in clinical alveolar proteinosis (Sakagami et al., 2009; Uchida et al., 2007). The capacity of host macrophages to repopulate after radiation injuries could have consequences in the bone marrow transplantation setting, in which remaining host antigen-presenting cells have been shown to contribute to graft-versus-host disease outcome (Hashimoto and Merad, 2011).

Because we now have the tools with which to distinguish monocytes and infiltrating macrophages from resident macrophages, we advocate for more stringent distinctions to be made between these two populations. We believe that this will be important in understanding tissue-resident macrophage homeostasis in humans subjected to various opportunistic (e.g., pathogenic infections) or iatrogenic (e.g., myeloablative procedures) insults, but more importantly, this will be critical to our understanding of the contribution of tissue-resident macrophages and BM-derived macrophages to tissue immunity, homeostasis, and repair.

EXPERIMENTAL PROCEDURES

Mice

The mouse strains used are described in the Supplemental Experimental Procedures.

Flow Cytometry

The detailed procedures, antibodies used, and gating schemes are described in the Supplemental Experimental Procedures.

Parabiosis

Parabiotic mice were generated as reported (Ginhoux et al., 2009) from age- and weight-matched CD45.2^{+} (C57BL/6 or *Ccr2*$^{-/-}$) and CD45.1^{+} (C57BL/6) mice that were between 6 and 8 weeks old. Consistent with prior publications (Ginhoux et al., 2009; Liu et al., 2007), circulating B cells and neutrophils achieved efficient (~50%) nonhost chimerism at 2 and 5 months.

Macrophage Depletion

The detailed protocols are described in the Supplemental Experimental Procedures.

Microbe-Induced Lung Inflammation

Mice were infected intranasally with 100 pfu of influenza strain A/Puerto Rico/8/34 (PR8) diluted in 25 μl PBS as previously described (Helft et al., 2012). Other Mice were intranasally instilled with 50 μg of high-molecular-weight polyinosinic-polycytidylic acid [Poly (I:C)] that was purchased from InvivoGen and reconstituted in normal saline (1 mg/ml).

RNA Isolation, Reverse Transcription, and Quantitative Real-Time PCR

The detailed protocols are described in the Supplemental Experimental Procedures.

Concomitant Csf1-R and Csf-2 Blockade

Blocking antibody to Csf-1R (clone AFS98) was produced from a hybridoma in house. Blocking antibody to Csf-2 (clone 22E9) was purchased from R&D. Anti-Csf-1R (600 μg) was infused intranasally on days −8, −6, −4, and −1, and 100 μg anti-Csf-2 was infused intranasally on days −7, −5, −2 prior to harvest.

Bone Marrow Adoptive Transfer

In order to achieve donor circulating chimerism without parabiosis or conditioning, we infused 7.5 × 10^{7} total BM cells from CD45.1^{+} mice into CD169$^{DTR/+}$ (CD45.2^{+}) animals.

BM and Fetal Liver Transplantation

CD45.1^{+} mice were irradiated (1,200 cGy, two split doses, 3 hr apart) in a Cesium Mark 1 irradiator (JL Shepperd & Associates) and then infused with 5 × 10^{6} BM cells from wild-type or *Csf-2r*$^{-/-}$ CD45.2^{+} cells or 1 × 10^{6} fetal liver cells from wild-type or *Csf1r*$^{-/-}$ animals. Mice were harvested and analyzed as detailed in the main text and figure legends.

Statistical Analyses

The unpaired Student's t test was used in all analyses, data in bar graphs are represented as means ± SEM, and statistical significance is expressed as follows: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; and n.s., not significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and Figures S1–S6.

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