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HIGHLIGHTED ARTICLE

Frontline Science: Mast cells regulate neutrophil homeostasis by influencing macrophage clearance activity

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Abstract

The receptor tyrosine kinase cKit and its ligand stem cell factor are essential for mast cells (MC) development and survival. Strains with mutations affecting the *Kit* gene display a profound MC deficiency in all tissues and have been extensively used to investigate the role of MC in both physiologic and pathologic conditions. However, these mice present a variety of abnormalities in other immune cell populations that can affect the interpretation of MC-related responses. C57BL/6 $Kit^{W\text{-}sh}$ are characterized by an aberrant extramedullary myelopoiesis and systemic neutrophilia. MC deficiency in $Kit^{W\text{-}sh}$ mice can be selectively repaired by engraftment with in vitro-differentiated MC to validate MC-specific functions. Nevertheless, the impact of MC reconstitution on other immune populations has never been evaluated in detail. Here, we specifically investigated the neutrophil compartment in primary and secondary lymphoid organs of C57BL/6 $Kit^{W\text{-}sh}$ mice before and after MC reconstitution. We found that, albeit not apparently affecting neutrophils phenotype or maturation, MC reconstitution of $Kit^{W\text{-}sh}$ mice restored the number of neutrophils at a level similar to that of wild-type C57BL/6 mice. In vitro and *ex vivo* experiments indicated that MC can influence neutrophil clearance by increasing macrophages' phagocytic activity. Furthermore, the G-CSF/IL-17 axis was also influenced by the presence or absence of MC in $Kit^{W\text{-}sh}$ mice. These data suggest that MC play a role in the control of neutrophil homeostasis and that this aspect should be taken into account in the interpretation of results obtained using $Kit^{W\text{-}sh}$ mice.

KEYWORDS
Kit (W-sh) mice, macrophages, mast cell, neutrophils, phagocytosis

1 | INTRODUCTION

Mast cells (MC) express the tyrosine kinase receptor cKit (CD117) at all stages of maturation and require the cKit ligand, stem cell factor (SCF), for their survival, maturation, and differentiation. Therefore, mice bearing mutations in the cKit gene have been extensively used in the past years to model MC deficiency. The $Kit^{W/W\text{-}V}$ mutation results in impaired tyrosine kinase activity of the cKit receptor, consequently leading to severe MC deficiency.[1] However, defective cKit signaling causes alterations in several other cell types, including melanocytes, neutrophils, interstitial cells of Cajal, and intraepithelial $\gamma\delta$T lymphocytes.[2,3] $Kit^{W/W\text{-}V}$ mice are also anemic, sterile, and prone to dermatitis and gastric disorders.[4–6] For such reasons, their use has been limited in favor of $Kit^{W\text{-}sh/W\text{-}sh}$ "sash" mice ($Kit^{W\text{-}sh}$ mice). The *W-sh* mutation is an inversion in chromosome 5,[7] including the regulatory elements critical to control the expression of the cKit gene in MC.[8] $Kit^{W\text{-}sh}$ mice are therefore MC deficient and display fewer abnormalities compared with $Kit^{W/W\text{-}V}$ mice. Indeed, despite having defects in melanocytes and interstitial cells of Cajal, $Kit^{W\text{-}sh}$ mice are fertile, not anemic[7,9] and not susceptible to gastric disorders.[10] Also, the frequencies of the majority of mature immune cell populations are not affected.[10] However, $Kit^{W\text{-}sh}$ mice evidence splenomegaly and expanded frequencies of megakaryocyte and myeloid cells

Abbreviations: BMMC, bone marrow-derived mast cells; CFSE, carboxyfluorescein diacetate succinimidyl ester; $Kit^{W\text{-}sh/W\text{-}sh}$, $Kit^{W\text{-}sh}$; MC, mast cells; SCF, stem cell factor; WT, wild-type

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populations, including neutrophils.[11] Aberrant extramedullary myelopoiesis characterized by the accumulation of $CD11b^+$ cells expressing intermediate and high levels of Ly6G has also been described in these mice.[12]

All these abnormalities must be taken into account when analyzing experiments performed in $Kit^{W\text{-}sh}$ mice, especially when focusing on innate immune responses. Indeed, both MC and neutrophils are critical for properly mounting acute inflammatory reactions in several disease settings. Importantly, it has been reported in murine models that MC can influence neutrophils recruitment during inflammation[13–15] and can modulate their effector functions.[16] This interaction is lacking in $Kit^{W\text{-}sh}$ mice; therefore, inflammatory responses might be altered not only as a consequence of MC deficiency "per se," but also in relation to the aberrant neutrophilia that characterizes these mice.

MC deficiency in $Kit^{W\text{-}sh}$ mice can be selectively repaired by adoptive transfer of bone marrow-derived in vitro-cultured MC (BMMC).[10] This approach has been used to specifically validate the biological functions of MC. However, to the best of our knowledge, the impact of MC reconstitution in the control of neutrophils frequency and homeostasis in $Kit^{W\text{-}sh}$ mice has never been addressed. Indeed, as the cKit receptor is expressed by hematopoietic stem cells, neutrophils' alterations may be a direct result of the genetic mutation in $Kit^{W\text{-}sh}$ mice, but it cannot be excluded that MC can directly influence their numbers and homeostasis. These two hypotheses are herein investigated.

2 | MATERIALS AND METHODS

2.1 | Mice

C57BL/6 mice and MC-deficient C57BL/6 $Kit^{W\text{-}sh/W\text{-}sh}$ ($Kit^{W\text{-}sh}$) were purchased from The Jackson Laboratory. Mice were maintained under pathogen-free conditions at the animal facility of Fondazione IRCCS Istituto Nazionale dei Tumori. All animal experiments were performed in accordance with the animal care and use committees of the institutes, and in accordance with the Italian Law (D.lgs 26/2014).

Single-cell suspensions of bone marrow and spleen were obtained by mechanical dissociation of organs through 70-mm-pore-size nylon filters. Red blood cells in spleen and blood samples were lysed with ACK lysing buffer (Lonza). Peritoneal cells were obtained injecting cold PBS supplemented with 3% FBS in the peritoneal cavity and dislodging any attached cell by massaging the peritoneum.

2.2 | Immunohistochemistry

Murine spleens were fixed in formalin and embedded in paraffin. Sections (5 μm) were stained with H&E (BioOptica) and evaluated by a pathologist. Slides were analyzed under an Axioscope A1 microscope equipped with Axiocam 503 Color camera (Zeiss).

2.3 | FACS analysis

Phenotype analysis by flow cytometry was performed on freshly isolated and on cultured cells after washing in buffer containing 2% BSA. Cell suspensions were incubated 10 minutes with FcR blocker (eBioscience), labeled for 15 minutes at 4°C with fluorochrome-conjugated monoclonal antibodies or appropriate isotype controls (all from eBioscience or BioLegend). The following anti-mouse mAbs were used: anti-FcεRI (PE), anti-CD117 (FITC), anti-CD11b (APC o FITC), anti-CD62L (PE), anti-CXCR2 (PE), anti-CXCR4 (PeCy5), anti-F4/80 (FITC), anti-Ly6G (BV421), anti-Ly6C (BV605), anti-IA/E (BV510), anti-MerTK (PE), and anti-Tim4 (APC).

For intracellular staining of Ly6G, cells were stained for surface markers, fixed with 2% PFA, and permeabilized with saponin (0.5% in PBS), before incubation with the desired antibody.

Samples were analyzed using FACSCalibur (BD Biosciences) and BD LSRII Fortessa (BD Biosciences) instruments and analyzed with the FlowJo software.

2.4 | Generation of BMMC and reconstitution of MC-deficient mice

Primary cultures of BMMC were obtained from the bone marrow of 6-week-old C57BL/6 mice. In vitro differentiation was induced by adding 20 ng/mL of IL-3 and SCF (PeproTech) in the culture medium for 5 weeks. The purity of BMMC was evaluated by flow cytometry as a percentage of FcεRI and cKit-positive cells and was usually more than 96%.

A total of 5×10^6 BMMC were injected i.p. into 8-week-old $Kit^{W\text{-}sh}$ mice to reconstitute the MC population (Rec $Kit^{W\text{-}sh}$) and used after 4 weeks together with age-matched C57BL/6 and $Kit^{W\text{-}sh}$ mice. MC reconstitution was confirmed by flow cytometry evaluating double-positive staining for FcεRI and cKit markers on peritoneal cells.

2.5 | Isolation of neutrophils and macrophages

Neutrophils were obtained from the bone marrow of 6-week-old C57BL/6 or $Kit^{W\text{-}sh}$ mice by positive selection magnetic cell separation using the Miltenyi Biotec anti-Ly6G-magnetic microbeads Kit (Miltenyi Biotec). Purity was evaluated by analyzing by flow cytometry the expression of the Ly6G (D1A8 clone) and CD11b surface markers. Cells were at least 97% double positive.

Peritoneal macrophages were obtained from peritoneal lavage of 6-week-old C57BL/6 mice using the Peritoneal Macrophage Isolation Kit (Miltenyi Biotec), following manufacturers' instruction.

2.6 | Neutrophil cell death assay

$Ly6G^+$ neutrophils were cultured in DMEM with 10% FBS or induced to apoptosis by culturing them in media without serum. To investigate cell survival capacity, cells were collected at different time points, stained with PI and Annexin V, and analyzed by flow cytometry.

2.7 | In vitro phagocytosis assay

$Ly6G^+$ cells isolated from bone marrow of C57BL/6 mice were suspended at 2×10^6 cell/mL in 10% RPMI, labeled for 10 minutes with 2.5 μmol/L APC-conjugated CFSE, washed twice and then incubated

in RPMI without serum for 20 hours at 37°C in 5% CO_2 to induce cell apoptosis. CFSE-labeled Ly6G$^+$ cells were added for 30 minutes to peritoneal macrophages at a 10:1 ratio in the presence or absence of cytochalasin D 1 μmol/L (inhibitor of phagocytosis). Flow cytometry was used to quantify percentages of F4/80-labeled macrophages that had engulfed CFSE-labeled cells. The percentage of phagocytosis was calculated based on the percentage of double-positive cells (corresponding to the percentage of binding and uptake) and by subtracting the percentage of double-positive cells in the presence of cytochalasin D (corresponding to the percentage of binding).

In some experiments, equal numbers of BMMC and macrophages were preincubated for 2 hours. Then, 10-fold numbers of CFSE-labeled Ly6G$^+$ cells were added, and phagocytosis assay was conducted in the presence or absence of cytochalasin D as described.

2.8 | ELISA assays

The amount of B cell activation factor (BAFF) and IL-6 released by neutrophils in response to LPS stimulation was assessed after 24 hours of cell stimulation using the specific BAFF ELISA assay (Boster Biological Technology, Ltd) and the IL-6 ELISA assay (eBioscience) following manufacturer's indications.

The serum levels of cytokines were evaluated by specific ELISA Kit assays. ELISA for SCF and G-CSF were from RayBiotech; TNF-α, IL-17, and IL-22 ELISA Kit assays were from eBioscience.

2.9 | Data handling and statistical analysis

Results are presented as mean ± SEM and data analysis was performed with the Prism GraphPad Software. For comparisons between two groups, the two-tailed unpaired and paired Student *t* tests were used. When multiple comparisons were necessary, data were analyzed with the one-way ANOVA test, and the Bonferroni correction was used as post hoc analysis. In all tests, P values < 0.05 were considered statistically significant.

3 | RESULTS

3.1 | Mast cell reconstitution reduces the frequency of myeloid cells in Kit$^{W\text{-}sh}$ mice

It is well known that, beyond MC deficiency, Kit$^{W\text{-}sh}$ mice show increased myelopoiesis in steady-state conditions compared with MC-competent wild-type (WT) mice.[11,12] To analyze whether Kit$^{W\text{-}sh}$ mice engraftment with in vitro-cultured bone marrow-derived MC (BMMC) could restore the frequency of myeloid cells, BMMC obtained from WT C57BL/6 mice were intraperitoneally transferred into Kit$^{W\text{-}sh}$ mice. Four weeks after MC reconstitution, we investigated by flow cytometry the frequency of CD11b$^+$ cells in bone marrow, spleen, and peripheral blood of C57BL/6 (B6), Kit$^{W\text{-}sh}$, and reconstituted Kit$^{W\text{-}sh}$ (Rec Kit$^{W\text{-}sh}$) mice (Fig. 1). Consistent with previous reports,[11,12,17] the relative frequency (Figs. 1A and B) and absolute numbers (Fig. 1C) of CD11b$^+$ myeloid cells were increased in the spleen (middle panel) and peripheral blood (lower panel), but not in the bone marrow (upper panel), of Kit$^{W\text{-}sh}$ mice as compared with WT C57BL/6 counterparts. After MC reconstitution, the frequency and numbers of CD11b$^+$ cells in the spleen and peripheral blood of Rec Kit$^{W\text{-}sh}$ mice decreased significantly, reaching almost the levels observed in C56BL/6 mice (Figs. 1A-C). The decrease of CD11b$^+$ cells in Rec Kit$^{W\text{-}sh}$ mice was not accompanied by changes in the morphology of the spleens, which still exhibited the gross (Fig. 1D) and microscopic (Fig. 1E) abnormalities typical of Kit$^{W\text{-}sh}$ mice.[11] Indeed, spleens of both Kit$^{W\text{-}sh}$ and Rec Kit$^{W\text{-}sh}$ mice showed enlargement of the red pulp and increase of megakaryocyte and myeloid elements.

3.2 | BMMC reconstitution of Kit$^{W\text{-}sh}$ mice reduces the number of neutrophils

Previous reports highlighted that the abnormal myelopoiesis in Kit$^{W\text{-}sh}$ mice was mainly due to the expansion of cells expressing Ly6G within the CD11b$^+$ compartment.[11,12] In particular, Nigrovic et al. found in Kit$^{W\text{-}sh}$ mice an increased frequency of neutrophils, identified using an anti Gr1 antibody able to recognize both Ly6G and Ly6C epitopes.[11] Further investigation by Michel et al. showed an accumulation of both CD11b$^+$/Ly6G^{Int}/Ly6C^{low} and CD11b$^+$/Ly6G^{Hi}/Ly6C^{low} subpopulations in the spleen of Kit$^{W\text{-}sh}$ mice.[12] Based on these pieces of evidence, we adopted the same gating strategy as used by Michel et al. to investigate if the percentages and absolute numbers of CD11b$^+$/Ly6G^{Int}/Ly6C^{low} and CD11b$^+$/Ly6G^{Hi}/Ly6C^{low} cells were recovered to steady-state levels in Kit$^{W\text{-}sh}$ mice following MC reconstitution.

The frequencies of these populations within CD11b$^+$ cells did not change between the analyzed groups of mice in the blood compartment (Figs. 2A and B), whereas in the spleen we observed, as expected,[12] an increase in the frequency of CD11b$^+$/Ly6G^{Int}/Ly6C^{low} among CD11b$^+$ cells (Figs. 2D-E). Remarkably, the analysis of absolute cell counts highlighted an accumulation of CD11b$^+$/Ly6G^{Hi}/Ly6C^{low} in the blood and spleen of Kit$^{W\text{-}sh}$ mice that returned to normal levels after MC reconstitution (Figs. 2C and F). Reconstitution with MC also reduced, but not normalized to WT levels, the total number of CD11b$^+$/Ly6G^{Int}/Ly6C^{low} in the spleen (Fig. 2F). We did not find any significant difference in the frequencies and counts of these two subpopulations in the bone marrow of C57BL/6, Kit$^{W\text{-}sh}$, and Rec Kit$^{W\text{-}sh}$ mice (Supplemental Fig. S1A).

High expression of Ly6G on CD11b$^+$ cells likely identifies neutrophils.[18] For deeper characterization of neutrophils-associated maturation and trafficking molecules, we analyzed by flow cytometry the expression of CD11b, CXCR2, CD62L, and CXCR4 on CD11b$^+$/Ly6G^{Hi}/Ly6C^{low} from bone marrow, spleen, and peripheral blood of C57BL/6, Kit$^{W\text{-}sh}$, and Rec Kit$^{W\text{-}sh}$ mice. We did not detect any difference in the expression of the investigated markers in CD11b$^+$/Ly6G^{Hi}/Ly6C^{low} from the bone marrow (Supplemental Fig. S1B). However, we observed a reduction of CD11b and CXCR2 expression in cells obtained from the peripheral blood and spleen of Kit$^{W\text{-}sh}$ mice when compared with those obtained from control mice. MC reconstitution of Kit$^{W\text{-}sh}$ mice did not affect the expression levels

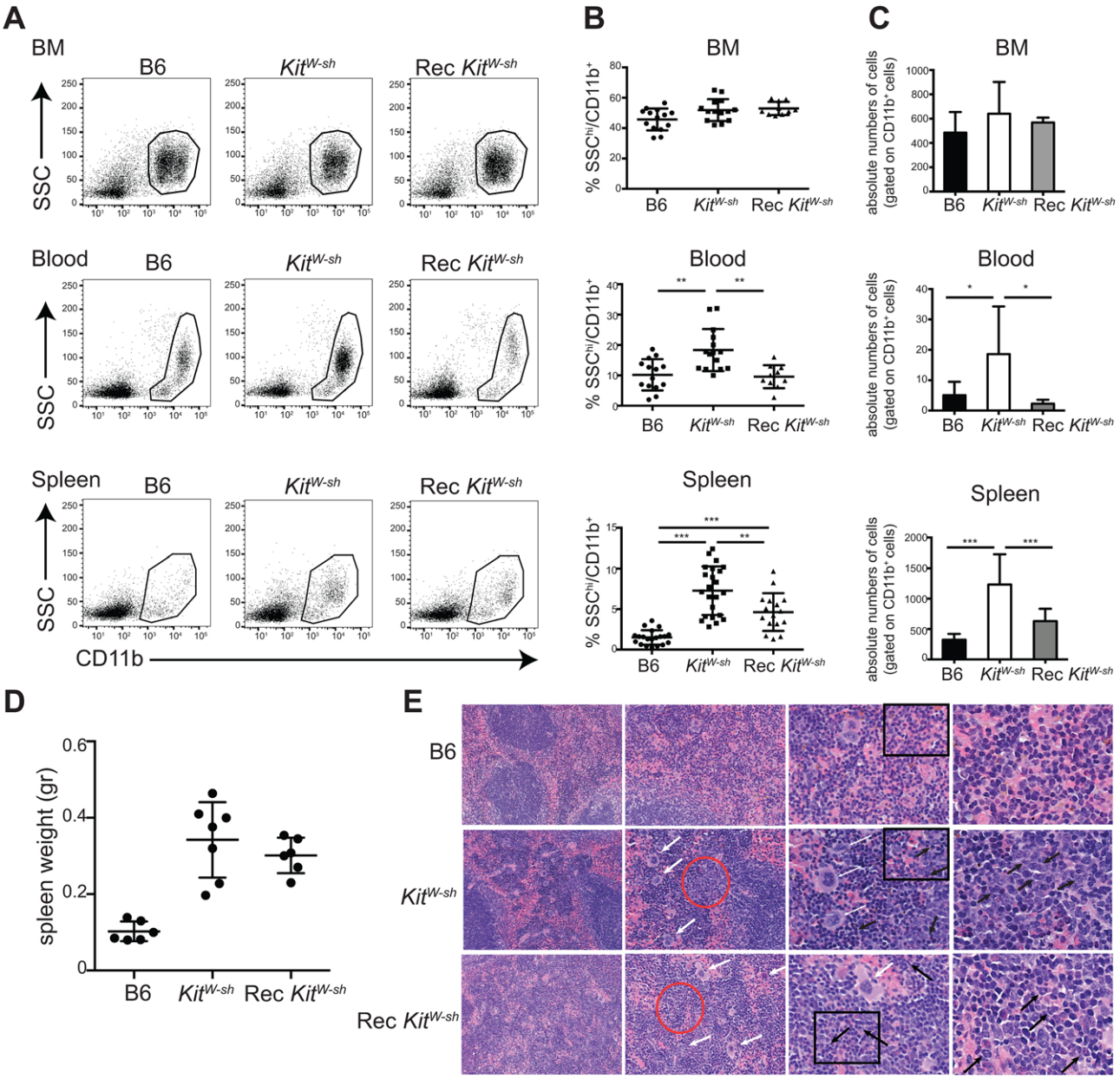

FIGURE 1 **Numbers of CD11b$^+$ cells are modified in Kit-deficient mice and recover after MC reconstitution.** Representative flow cytometry plots (A), percentages (B), and absolute numbers (C) of CD11b$^+$ cells in bone marrow (BM), blood, and spleen of B6, *Kit*$^{W\text{-}sh}$, and *Kit*$^{W\text{-}sh}$ mice reconstituted with in vitro-derived BMMC (Rec *Kit*$^{W\text{-}sh}$). Percentages are depicted as values from individual mice; bar indicates mean ± SEM. ANOVA followed by Bonferroni correction; $^*P < 0.05$, $^{**}P < 0.005$, $^{***}P < 0.001$ (B6, $n = 14$, *Kit*$^{W\text{-}sh}$; $n = 16$; Rec *Kit*$^{W\text{-}sh}$, $n = 16$). (D) Quantitation of spleen weight from B6, *Kit*$^{W\text{-}sh}$, and Rec *Kit*$^{W\text{-}sh}$ mice (B6, $n = 6$, *Kit*$^{W\text{-}sh}$, $n = 7$; Rec *Kit*$^{W\text{-}sh}$, $n = 6$; E). H&E staining of B6, *Kit*$^{W\text{-}sh}$, and Rec *Kit*$^{W\text{-}sh}$ spleens at different magnifications (from left: ×100, ×200, ×400, and further magnification of areas included in black rectangles). Red circles highlight areas of abnormal red pulp expansion. Arrowheads show megakariocytes (white) or monocytic and granulocytic elements (black)

of these markers in both splenic and circulating neutrophils (Fig. 2G). On the contrary, we did not detect any differences in the relative mean fluorescence intensities of CD62L and CXCR4 on splenic either peripheral blood Ly6G^{Hi}/Ly6C^{low} cells among the three groups of animals. These results indicate that neutrophils in the periphery of Kit$^{W\text{-}sh}$ have an immature phenotype, regardless of MC reconstitution.

Altogether, our results suggested that MC reconstitution can reverse the neutrophilia observed in Kit$^{W\text{-}sh}$ without affecting neutrophil maturation status. Such evidence suggests that MC can directly or indirectly control neutrophils number, whereas their immature phenotype is likely merely due to an intrinsic defect associated with the cKit mutation rather than due to MC deficiency, as previously suggested.[12]

3.3 | Neutrophils from WT and Kit$^{W\text{-}sh}$ mice are equally responsive

We next sought to determine whether neutrophils from Kit$^{W\text{-}sh}$ mice had also functional defects. CD11b$^+$/Ly6G$^+$ cells were purified from the bone marrow of C57BL/6 and Kit$^{W\text{-}sh}$ mice (Supplemental Fig. S2).

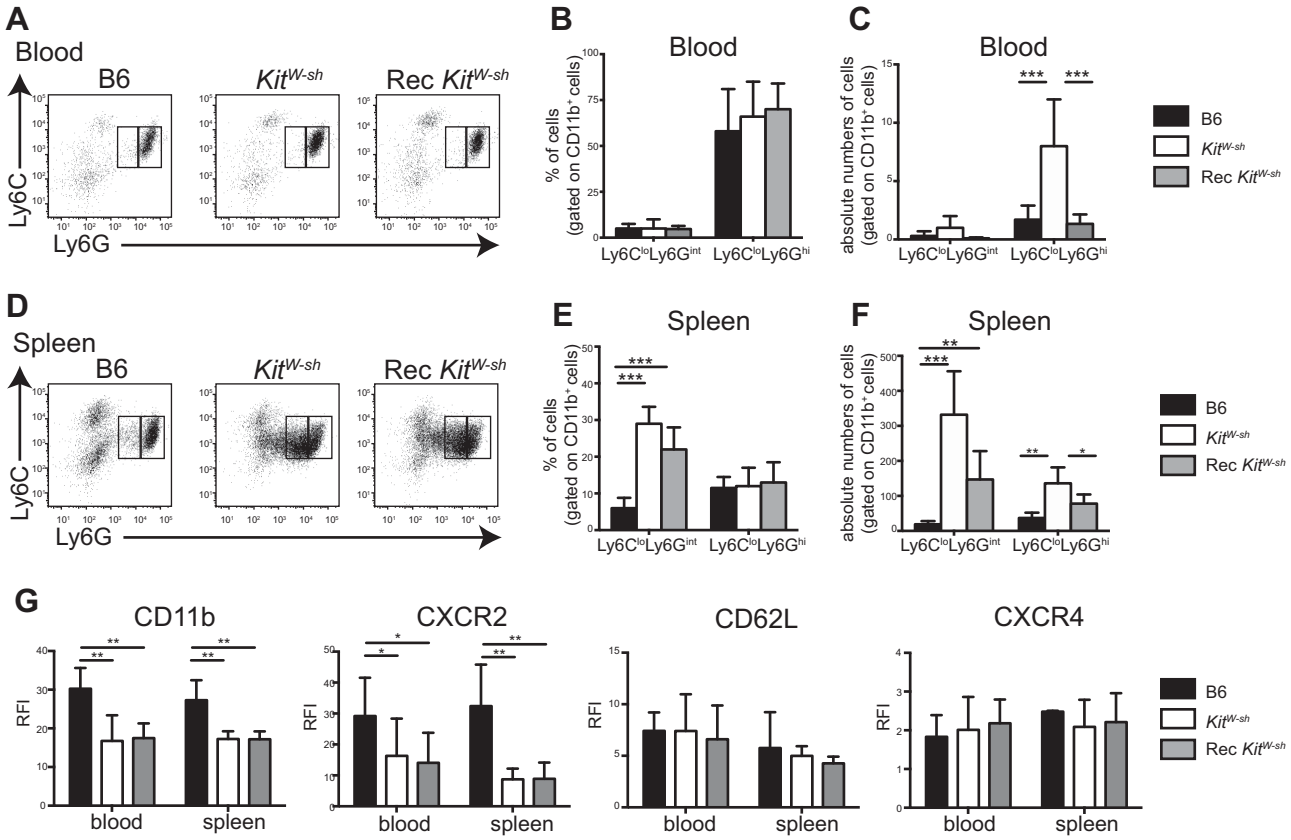

FIGURE 2 **Counts and phenotype of neutrophils are different in WT, *Kit*$^{W\text{-}sh}$, and reconstituted *Kit*$^{W\text{-}sh}$ mice** Representative flow cytometry plots (A, D), percentages (B, E), and absolute numbers (C, F) of Ly6C and Ly6G cells within CD11b$^+$ cells (gated as in Fig. 1) obtained from the blood and spleen of B6, *Kit*$^{W\text{-}sh}$, and Rec *Kit*$^{W\text{-}sh}$ *mice*. (G) Surface expression of CD11b, CXCR2, CD62L, and CXCR4 of Ly6C^{low}/Ly6G^{hi} cells, gated as in A. Histogram reports relative fluorescence intensity (RFI), measured as a ratio of mean fluorescence intensity between the sample and a control sample stained with the appropriate isotype antibody. ANOVA followed by Bonferroni correction; $^*P < 0.05$, $^{**}P < 0.005$, $^{***}P < 0.001$ (B6 $n = 14$, *Kit*$^{W\text{-}sh}$; $n = 16$; rec *Kit*$^{W\text{-}sh}$, $n = 16$)

Giemsa staining did not show significant differences in the morphology of neutrophils from the two strains (Fig. 3A). We then cultured purified neutrophils in complete media or under the apoptotic condition, modeled by 0% of serum. No significant differences in the number of viable and dead cells were noted in both culture settings (Fig. 3B), suggesting that survival capacities of neutrophils from WT and Kit$^{W\text{-}sh}$ mice are similar. Moreover, stimulation with LPS induced comparable upregulation of CD11b (Fig. 3C) and downregulation of CD62L (Fig. 3D), as well as the comparable release of IL-6 (Fig. 3E) and BAFF (Fig. 3F) in neutrophils from both Kit$^{W\text{-}sh}$ and C57BL/6 mice. Therefore, neutrophils from WT and Kit$^{W\text{-}sh}$ mice are similarly functional.

3.4 | The presence of MC influences the G-CSF/IL-17 axis

Data collected so far highlighted differences in the frequency and maturation of neutrophils in the secondary lymphoid organs of Kit$^{W\text{-}sh}$ mice. Moreover, reconstitution experiments suggested that MC could directly influence the number of neutrophils in steady-state conditions.

To understand the mechanisms involved in the MC-dependent control of neutrophils numbers, we first investigated the levels of cytokines involved in myelopoiesis and myeloid cell differentiation in the sera of C57BL/6, Kit$^{W\text{-}sh}$, and Rec Kit$^{W\text{-}sh}$ mice. G-CSF is the major driver of neutrophil proliferation and acts in a complex network with other cytokines, such as IL-17, IL-22, and TNF-α, to regulate neutrophil numbers.[19] The lack of MC was associated with higher levels of SCF, TNF-α, and IL-22 in the sera of Kit$^{W\text{-}sh}$ mice with respect to C57BL/6 animals, but reconstitution did not change them. On the contrary, serum levels of G-CSF and IL-17 were increased in Kit$^{W\text{-}sh}$ mice and returned to physiologic levels after MC reconstitution (Fig. 4), suggesting that MC can be important in controlling the levels of cytokines regulating myelopoiesis.

3.5 | MC enhance the ability of macrophages to clear neutrophils in vitro

To find additional mechanisms responsible for neutrophilia in Kit$^{W\text{-}sh}$ mice, we asked whether MC could regulate the route of clearance of neutrophils. As MC have phagocytic activity,[20–22] we first

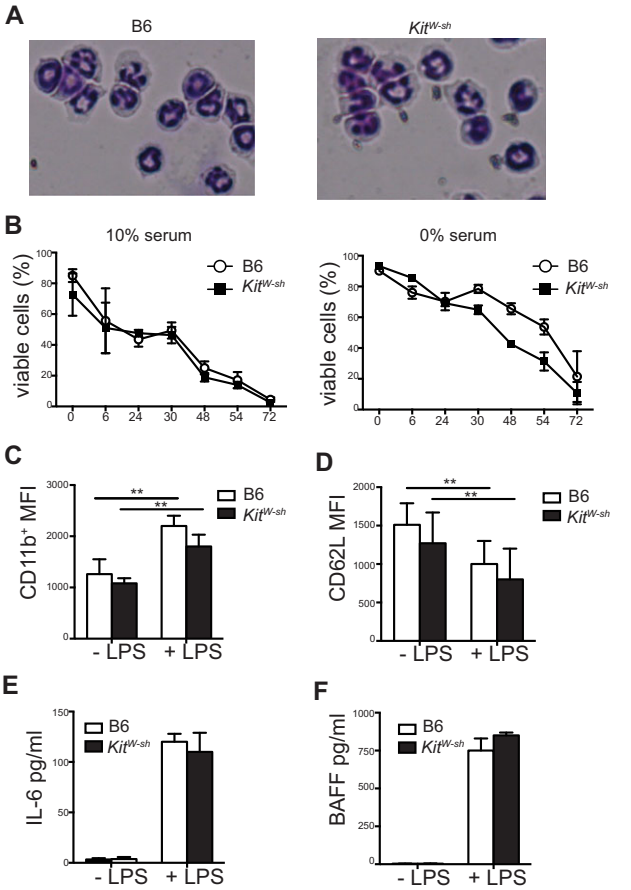

FIGURE 3 **Bone marrow-derived neutrophils from *Kit*$^{W\text{-}sh}$ mice are active.** (A) Giemsa staining of Ly6G$^+$ cells freshly purified from bone marrow of B6 and *Kit*$^{W\text{-}sh}$ mice. (B) Frequency of viable (PI-negative, annexin V-negative) Ly6G$^+$ cells purified from bone marrow and cultured in media with 10% or 0% FBS for 6, 24, 30, 48, 54, and 72 hours. Expression of CD11b (C) and of CD62L (D), or secretion of IL-6 (E) and BAFF (F) by Ly6G$^+$ cells obtained from B6 and *Kit*$^{W\text{-}sh}$ mice and stimulated or not with LPS for 30 minutes. Data are representative of three independent experiments. Paired Student *t* test; $^{**}P < 0.005$

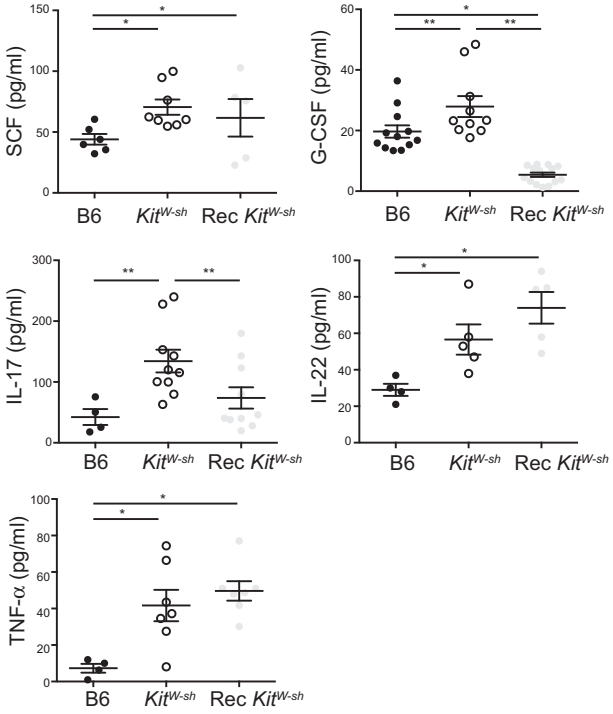

FIGURE 4 **Hematopoietic and proinflammatory cytokines are altered in Kit$^{W\text{-}sh}$ mice.** Levels of SCF, G-CSF, TNF-α, IL-17, and IL-22 measured by specific ELISA assays on sera of B6, Kit$^{W\text{-}sh}$, and Rec Kit$^{W\text{-}sh}$. ANOVA followed by Bonferroni correction; $^*P < 0.05$, $^{**}P < 0.005$ (B6, $n = 14$, *Kit*$^{W\text{-}sh}$; $n = 16$; Rec *Kit*$^{W\text{-}sh}$, $n = 16$)

hypothesized that they could directly phagocyte neutrophils. Therefore, we performed time-lapse analyses, following for 30 minutes cocultures between BMMC and viable or apoptotic CFSE-labeled Ly6G$^+$ cells, purified from the bone marrow of WT mice (Supplemental Fig. S3A), and quantified the engulfment of fluorescent cells by flow cytometry (Supplemental Fig. S3B). We observed MC-dynamic membrane ruffling with the concomitant rapid recruitment of neutrophils in close proximity to MC (Supplemental Fig. S3A). However, the level of neutrophils phagocytosis by MC was very low and even lower in the case of apoptotic neutrophils (Supplemental Fig. S3B).

Because the low capacity of neutrophil uptake by MC excluded their direct role in neutrophils clearance, we investigated if MC could influence the turnover of neutrophils by affecting the activity of specialized phagocytic cells. As macrophages are the most important scavengers of neutrophils in vivo,[23] we first tested the capacity of macrophages from both C57BL/6 and Kit$^{W\text{-}sh}$ mice of clearing neutrophils *ex vivo*. As shown in Fig. 5A, peritoneal macrophages isolated from C57BL/6 or Kit$^{W\text{-}sh}$ mice similarly phagocytized apoptotic CFSE-labeled neutrophils within 30 minutes of coculture (Fig. 5A). Phagocytic activity of C57BL/6 macrophages was also comparable against neutrophils purified from either WT or Kit$^{W\text{-}sh}$ mice (Fig. 5B). However, when BMMC were added in cocultures with macrophages and Ly6G$^+$ cells, the numbers of CFSE-positive macrophages increased significantly (Figs. 5A and B), suggesting that, at least in vitro, MC have the potential to enhance neutrophil clearance by macrophages. This effect was neutrophil specific, as macrophage phagocytosis of apoptotic B cells was unaltered by the presence or absence of MC in the coculture (Fig. 5C).

The MC-dependent increase of macrophages phagocytic capacity was corroborated by flow cytometry analysis of specific surface markers known to play crucial roles in the phagocytosis process. Even if the presence of MC in the culture did not affect the levels of CD11c, MerTK, and CD206 on peritoneal macrophages, it increased their expression of Tim4 (Fig. 5D), a phosphatidylserine receptor essential for the maintenance of phagocytosis of macrophages.[24]

3.6 | Neutrophil clearance by macrophages is altered in Kit$^{W\text{-}sh}$ mice after MC reconstitution

The above-described capability of MC to increase macrophages phagocytosis in vitro suggested the possibility that in MC-deficient Kit$^{W\text{-}sh}$ mice, macrophages could have a defect in neutrophil clearance. Therefore, we compared the capacity of macrophages with phagocytize neutrophils in C57BL/6, Kit$^{W\text{-}sh}$, and Rec Kit$^{W\text{-}sh}$ mice in vivo. First of all, to exclude that the accumulation of neutrophils in Kit$^{W\text{-}sh}$ mice was due to diminished numbers of tissue-resident macrophages, we

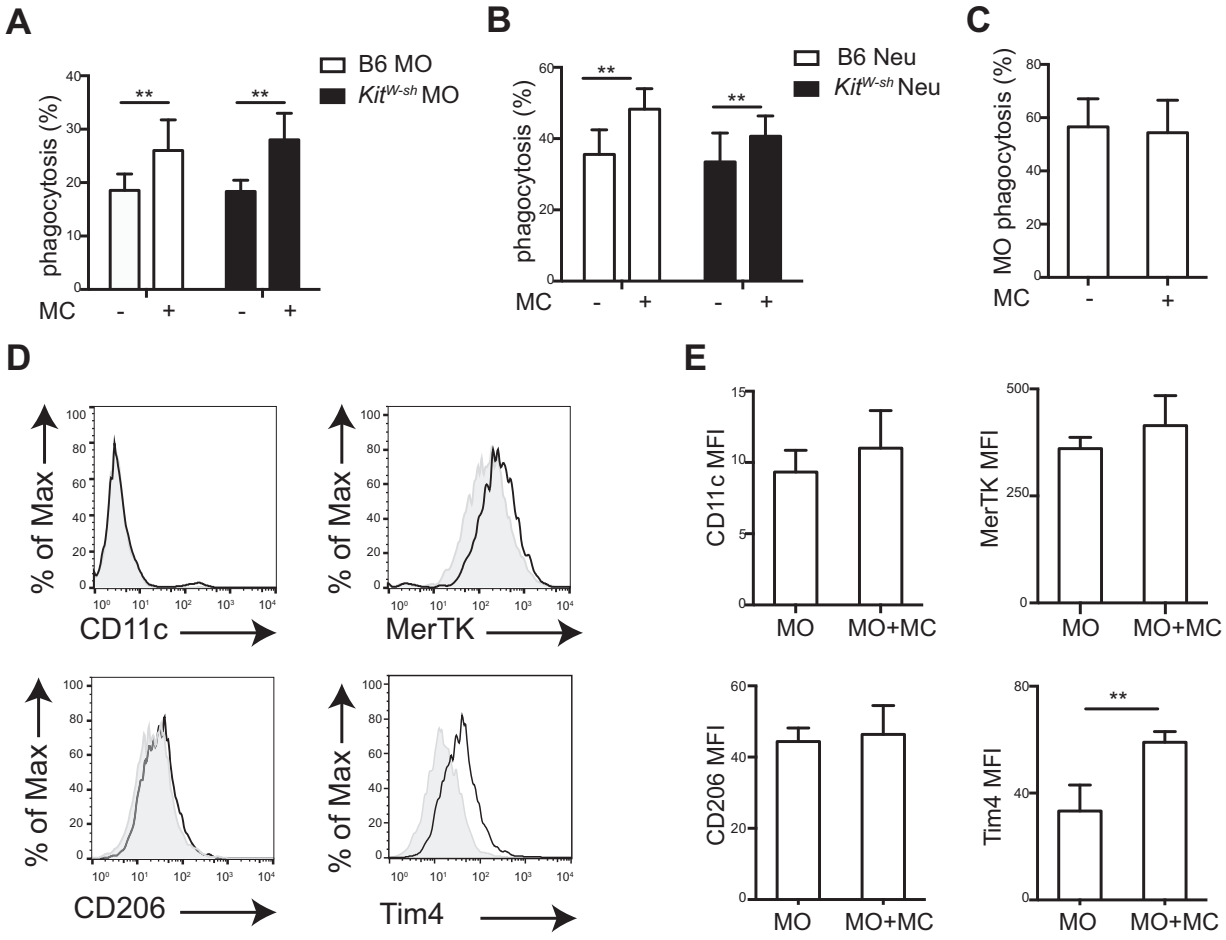

FIGURE 5 **MC specifically increase the capacity of macrophages to engulf neutrophils**. Peritoneal macrophages (MO) were incubated with CFSE-labeled Ly6G$^+$ cells in the presence or absence of BMMC and assessed for their ability to phagocytize neutrophils. (A) Comparison of the capacity of B6 and *Kit*$^{W\text{-}sh}$ macrophages with engulf neutrophils purified from B6 mice. (B) Percentages of macrophages (from B6 mice) that have engulfed neutrophils purified from B6 or *Kit*$^{W\text{-}sh}$ mice. (C) Macrophage ability to phagocytize apoptotic B cells. (A-C) Where indicated, BMMC were added during the coculture. Representative histograms (D) and MFI (E) of surface levels of CD11c, CD206, MerTK, and Tim4 on macrophages in coculture with MC. Data are representative of at least three independent experiments. Paired Student *t* test; **, $P < 0.005$

determined the percentages and absolute numbers of these cells in all three groups of mice. No differences were detected in the frequencies of CD11b$^+$/F480$^+$/MHCII$^+$/Ly6G$^-$ macrophages in the bone marrow (Fig. 6A), whereas we observed a slight increase in the number of splenic CD11b$^+$ F480$^+$/MHCII$^+$/Ly6G$^-$ macrophages in Kit$^{W\text{-}sh}$ and Rec Kit$^{W\text{-}sh}$ mice (Fig. 6B). Nevertheless, macrophages from both bone marrow and spleen of Rec Kit$^{W\text{-}sh}$ mice expressed the highest levels of Tim4 (Fig. 6C).

We then assessed the level of neutrophil clearance by macrophages in vivo by measuring the intracellular expression of the neutrophil-specific marker Ly6G in macrophages isolated from the bone marrow and spleen of C57BL/6, Kit$^{W\text{-}sh}$, and Rec Kit$^{W\text{-}sh}$ mice (Fig. 7). This technique is used to quantify the numbers of macrophages that had engulfed neutrophils in tissues under steady-state conditions.[25] Given the need to evaluate intracellular positivity for Ly6G, macrophages were gated as CD11b$^+$/F480$^+$/Ly6C$^-$/MHC^{Hi} (Supplemental Fig. S4). To be certain of not including Ly6G$^+$ cells within the gate, the same samples were in parallel analyzed for surface expression of Ly6G and were found negative (Supplemental Fig. S5). Flow cytometry analysis revealed a significant decrease in the percentages of Ly6G intracellular expression in CD11b$^+$/F480$^+$/Ly6C$^-$ macrophages recovered from the bone marrow of Kit$^{W\text{-}sh}$ mice as compared with WT mice (Figs. 7A-B), thus indicating a reduced capacity to ingest neutrophils in the absence of MC. Of note, Ly6G intracellular expression was restored to the level of WT mice in macrophages isolated from the bone marrow of Rec Kit$^{W\text{-}sh}$ mice (Fig. 7B). Even if we noted no differences in intracellular Ly6G expression in splenic macrophages from both C57BL/6and Kit$^{W\text{-}sh}$ mice, this was significantly increased when macrophages were analyzed from the spleen of Rec Kit$^{W\text{-}sh}$ mice (Figs. 7C-D).

Altogether, these data highlight a role for MC in influencing the ability of macrophages to clear neutrophils, a mechanism that can partly account for the neutrophilia observed in MC-deficient Kit$^{W\text{-}sh}$ mice and its reversion following MC reconstitution.

4 | DISCUSSION

Mice with abnormalities affecting the cKit gene show a profound MC deficiency and therefore have been extensively used to analyze the role of MC in several biological settings. However, both the

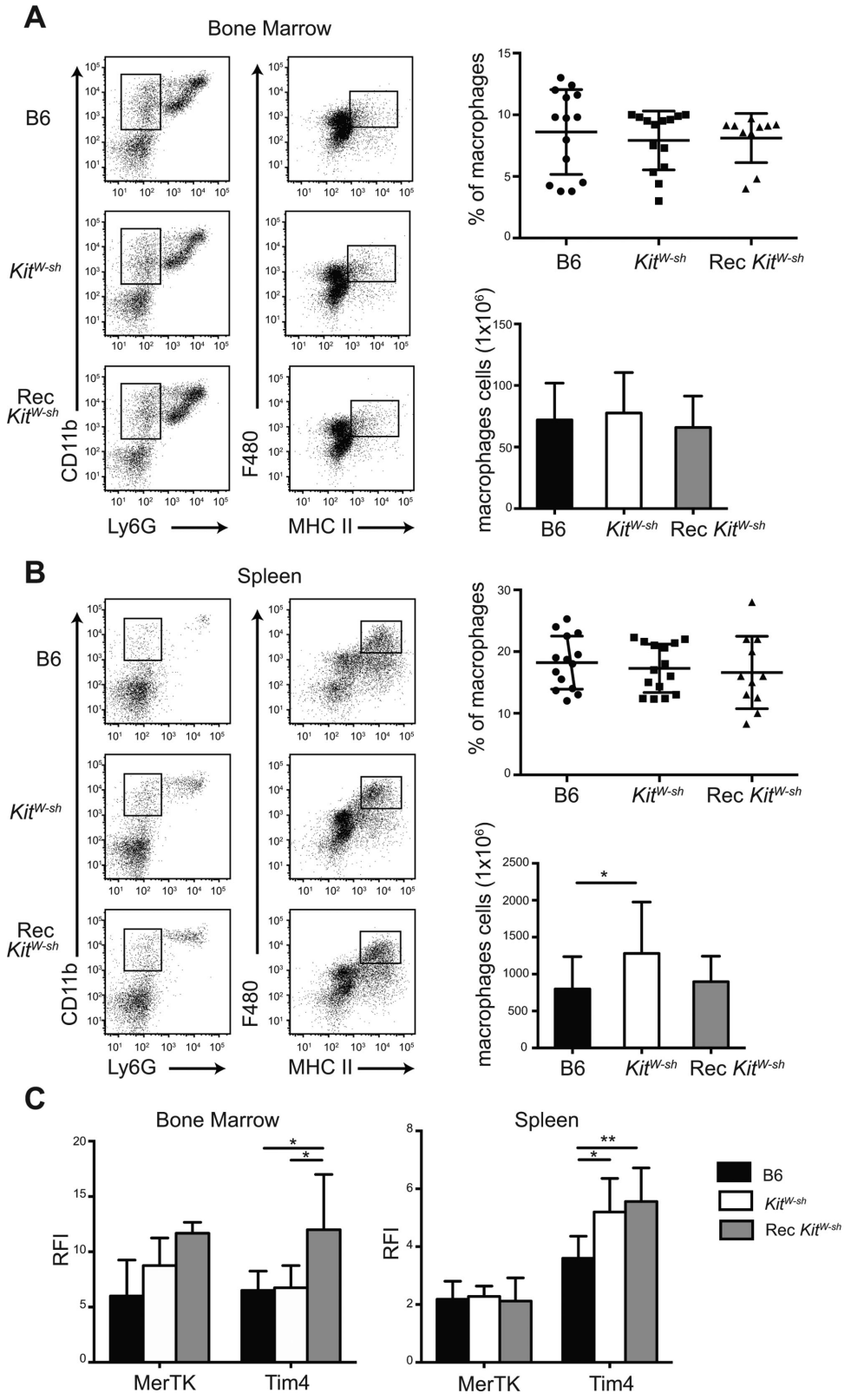

FIGURE 6 **Counts and phenotype of macrophages do not vary between B6, *Kit*$^{W\text{-}sh}$, and reconstituted *Kit*$^{W\text{-}sh}$ mice.** Gate strategy, percentages, and numbers of macrophages identified as CD11b^{+}/Ly6G^{-}/F4/80^{Hi}/MHC-II^{Hi} in bone marrow (A) and spleen (B) of B6, *Kit*$^{W\text{-}sh}$, and Rec *Kit*$^{W\text{-}sh}$ mice. Percentages are depicted as values from individual mice; bar indicates mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.005$, $^{***}P < 0.001$ (B6, $n = 14$, *Kit*$^{W\text{-}sh}$; $n = 15$; Rec *Kit*$^{W\text{-}sh}$, $n = 11$). (C) MerTK and Tim4 expression on macrophages identified as in A. Histogram reports RFI measured as a ratio of MFI between the sample and a control sample stained with the appropriate isotype antibody. ANOVA followed by Bonferroni correction; $^{*}P < 0.05$, $^{**}P < 0.005$

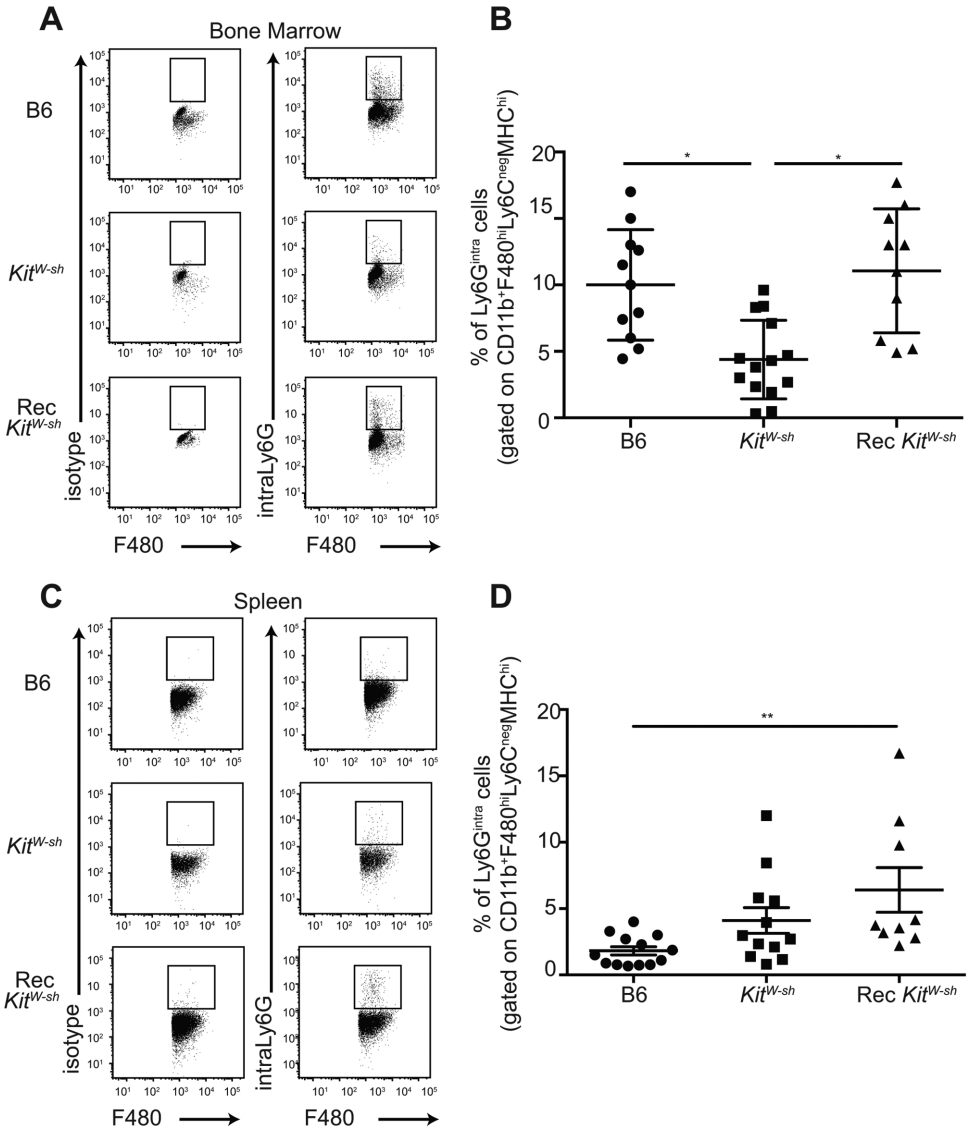

FIGURE 7 **Differences in neutrophils clearance by macrophages detected among B6, *Kit*$^{W\text{-}sh}$, and reconstituted *Kit*$^{W\text{-}sh}$ mice**. Extent of neutrophil clearance by macrophages in the spleen and bone marrow of mice under steady state. Representative dot plots and percentages of macrophages positive for intracellular Ly6G in the bone marrow (A-B) and spleen (C-D) of B6, *Kit*$^{W\text{-}sh}$, and Rec *Kit*$^{W\text{-}sh}$ mice. Percentages are depicted as values from individual mice; bar indicates mean ± SEM. ANOVA followed by Bonferroni correction; $^{*}P < 0.05$, $^{**}P < 0.005$ (C57BL/6, $n = 13$; W^{sh}; $n = 12$; W^{sh} rec, $n = 10$)

Kit$^{W/W\text{-}V}$ and Kit$^{W\text{-}sh}$ models are characterized by several other phenotypic abnormalities that may account for differences in the biological responses in both steady-state and pathologic conditions. It is not surprising that the continuous discovery of MC-unrelated abnormalities in Kit-mutant mice incites criticism and leads investigators to search for alternative Kit-independent animal models to study MC effector functions. However, a "gold-standard" mouse model with a selective and complete MC deficiency and absence of unrelated alterations is still lacking. Most of the Kit-independent models harbor a Cre recombinase under the control of MC-specific genes (i.e., Mcpt5, Cpa3, and FcεRIβ).[26] These mice can be constitutively depleted of MC (as in the case of Cpa3Cre, Cpa3-Cre Mcl1fl/fl, and Mcpt5-Cre R-DTA) or deletion can be induced by the injection of diphtheria toxin (Mcpt5-Cre, iDTR, and RMB mice).[27,28] In contrast to Kit-mutant mice, these MC-deficient animals show fewer hematological abnormalities but present other limitations including incomplete or selective MC depletion (i.e., mucosal rather than connective MCs), absolute or transient reduction in basophil numbers or functionality, and possible defects in other immune cells that again may affect interpretation of results.[26] Of note, although a number of in vivo MC functions have been confirmed in these newer Kit-independent MC-deficient mice, discordant results have also emerged suggesting a redundant or absent role for MC in models of arthritis, experimental autoimmune encephalomyelitis, or cutaneous contact hypersensitivity (reviewed in ref.26). Therefore, the choice of the suitable MC-deficient mouse model for in vivo experiments must take into account strain-specific advantages and defects.

The C57BL/6 Kit$^{W\text{-}sh/W\text{-}sh}$ (Kit$^{W\text{-}sh}$ strain), commonly used by several research groups, is characterized by splenomegaly and aberrant extramedullary myelopoiesis, resulting in the accumulation of

immature myeloid cells and neutrophils.[11] Considering the capability of MC to coordinate the trafficking of neutrophils in response to several inflammatory and infectious conditions,[14–16] it is crucial to keep in mind these defects in the myeloid compartment when interpreting results obtained with Kit$^{W\text{-}sh}$ mice. The possibility of selectively reverting MC deficiency in these mice through the adoptive transfer of in vitro-differentiated MC has been widely used as an experimental approach to validate their function and exclude the contributions of other Kit-dependent cell lineages. However, to the best of our knowledge, it has not been previously formally addressed whether the defects in the myeloid compartment of Kit$^{W\text{-}sh}$ mice can be reverted by MC reconstitution, or are irreversible. Indeed, due to the early expression of cKit in hematopoietic stem cells, its mutation can affect not only SCF-dependent MC differentiation and maturation but also other hematopoietic lineages. In a recent study, a single-cell transcriptional profile of bone marrow-derived hematopoietic stem cells and progenitor cells from a Kit-mutant W41/W41 mouse (characterized by a missense mutation in the kinase domain of the Kit coding sequence that causes a partial loss of protein function[29]) revealed that impaired cKit signaling exerts global effects on hematopoietic lineages with reduced MC and basophil progenitor cluster and a concomitant increase of neutrophil progenitors.[29]

Nevertheless, albeit this evidence suggests that the increased frequency of circulating and splenic neutrophils observed in Kit$^{W\text{-}sh}$ mice could be directly related to a defect in the cKit-SCF signaling, here we show that the levels of CD11b^{+}/Ly6G^{Hi}/Ly6C^{low} neutrophils return similar to the ones observed in WT mice after MC reconstitution. These results unravel a previously unreported role of MC in the regulation of homeostatic levels of neutrophils. We describe two mechanisms by which MC engraftment might directly or indirectly restore the frequency of neutrophils in Kit$^{W\text{-}sh}$ mice to levels similar to the ones observed in WT mice: (i) reduction of systemic levels of G-CSF and IL-17 and (ii) enhancement of macrophage-mediated neutrophil phagocytosis.

In steady state, the frequency of circulating neutrophils is tightly controlled by the coordinated regulation of granulopoiesis and mobilization from the bone marrow and by the clearance of senescent neutrophils. G-CSF is a master neutrophil-mobilizing cytokine and a major regulator of neutrophil homeostasis[19] acting in a coordinate with other cytokines, including IL-23 and IL-17. Here, we found that G-CSF and IL-17 levels are constitutively elevated in the sera of Kit$^{W\text{-}sh}$ mice compared with age-matched WT controls, a finding consistent with a condition of exacerbated myelopoiesis, and reduced following MC reconstitution.

In addition, we observed that bone marrow and splenic macrophages increase the uptake of neutrophils after MC reconstitution, an observation corroborated by in vitro data. After MC reconstitution of Kit$^{W\text{-}sh}$ mice, macrophages display increased expression of molecules known to play a significant role in phagocytosis such as MerTK and Tim4.[24] Though the identification of a mechanism behind this effect was out of the scope of this article, it appears to be specific for neutrophils as we did not find differences in the phagocytosis of B cells by macrophages in the presence of MC, at least in vitro.

Of note, as previously reported,[12] the accumulation of neutrophils is observed in the spleen and blood, but not in the bone marrow, of Kit$^{W\text{-}sh}$ mice. However, phagocytosis of macrophages in the bone marrow is reduced in Kit$^{W\text{-}sh}$ mice and is restored to WT levels after MC reconstitution, whereas in the spleen of reconstituted Kit$^{W\text{-}sh}$ mice, it is even higher than in WT. To explain this apparent discrepancy, we hypothesize two possible scenarios:

(i) In homeostatic conditions, MCs can regulate the rate of neutrophils clearance by sustaining macrophage activity mainly in the bone marrow, and not in the periphery. Therefore, in the bone marrow of Kit$^{W\text{-}sh}$ mice, defective neutrophil phagocytosis by macrophages, as a consequence of MCs absence, might result in further recirculation and accumulation of neutrophils in the spleen and blood. This eventuality has been suggested a few years ago[30] and could also be supported by the reduced levels of CXCR2 found in neutrophils from Kit$^{W\text{-}sh}$ and Rec Kit$^{W\text{-}sh}$ mice;

(ii) In Kit$^{W\text{-}sh}$ mice, the defects in the cKit gene lead to extramedullary hematopoiesis responsible for the accumulation of immature progenitors and myeloid subpopulation in the spleen, whereas the bone marrow is not affected.[12] MC reconstitution increases phagocytic activity of macrophages against Ly6G^{Hi} cells both in the spleen and bone marrow of Kit$^{W\text{-}sh}$ mice, likely compensating for the abnormal extramedullary production of neutrophils in the periphery of Kit$^{W\text{-}sh}$ mice[12] and restoring their numbers to WT levels. On the other hand, CD11b^{+}/Ly6G^{Int}/Ly6C^{low} cells, which are also continuously produced as a consequence of the c-Kit defects, cannot be normalized after reconstitution. In addition, the increased engulfment of neutrophils can also explain the reduced production of G-CSF in Rec Kit$^{W\text{-}sh}$, at levels even lower than those in WT mice. In fact, it has been described that after phagocytosis of neutrophils, macrophages can reduce their production of IL-23, a key promoter of IL-17 expression by $\gamma\delta$ or unconventional $\alpha\beta$ T cells, this latter cytokine a positive regulator of G-CSF levels. The activation of this negative feedback on granulopoiesis via the "IL-23–IL-17–G-CSF axis" [31] may further counterbalance the cKit intrinsic exacerbated myelopoiesis observed in Kit$^{W\text{-}sh}$ mice by reduced neutrophil mobilization.[32]

The synergistic contribution of MC and macrophages in the control of immune responses has been previously studied mainly in the context of inflammation, such as cardiovascular and metabolic diseases[33] and infections.[34] In a recent report, it was shown that MC, through the release of IL-4, reduce the capacity of peritoneal macrophages to promote bacterial clearance by inhibiting phagocytosis in a sepsis model.[35] This is apparently in contrast with our findings demonstrating that macrophage phagocytosis of neutrophils is improved in the presence of MC, but conversely suggests that different pathways and/or inflammatory versus homeostatic signals may differentially regulate MC modulation of macrophage functions. The importance of an MC-macrophage axis in regulating neutrophil homeostasis has never been addressed, and further studies are required to understand the significance of this result beyond our observations in Kit$^{W\text{-}sh}$ mice. Nevertheless, data from different MC-deficient mouse strains suggest that

this could be a conserved mechanism. In fact, a modest but significant increase in the number of splenic neutrophils has been reported in MC-deficient Cpa3-Cre; Mcl-1flfl^{+}[27] but not in other Kit-independent mouse models of MC deficiency, such as the Cre-Master Cpa3Cre/+, neither in Mcpt5-Cre iDTR.[35] Considering that incomplete or selective MC depletion can occur in these strains, the issue deserves further investigation.

Interestingly, the fact that MC reconstitution cannot revert neutrophils' immature status, characterized by low levels of CD11b and CXCR2, suggests that the limited maturation of circulating neutrophils in Kit$^{W\text{-}sh}$ mice is intrinsically linked to the W-sh mutation, as previously suggested.[12] Also, MC reconstitution did not completely restore the number of splenic CD11b^{+}/Ly6G^{Int}/Ly6C^{low} cells. The finding that these immature myeloid populations persist after MC reconstitution should be taken into account when utilizing the Kit$^{W\text{-}sh}$ mouse strain.

In conclusion, our analysis clarifies the effect of MC reconstitution in the regulation of the neutrophil compartment in Kit$^{W\text{-}sh}$ mice. Although the mechanisms remain to be clarified, our data suggest that MC reconstitution can affect both the main cytokine axis involved in the control of granulopoiesis and efferocytosis responsible for neutrophil homeostasis in vivo. We also uncover a previously undescribed potential role for MC in controlling neutrophil clearance in homeostatic conditions by prompting the phagocytic ability of macrophages. Further studies will be required to determine how the MC-macrophage axis can regulate neutrophil numbers and function in physiologic and pathologic conditions.

AUTHORSHIP

E.J. and F.D.: acquisition and analysis of data, drafting of the manuscript; R.M., C.D.S., F.V, and V.C.: technical support; L.D., C.T., and P.S.: technical support and interpretation of data. M.P.C. and C.P.: study design and obtained funding. B.F.: study supervision, acquisition and analysis of data, drafting of the manuscript. E.J. and F.D. share first authorship of this article.

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CONFLICTS OF INTEREST

No potential conflicts of interest were disclosed.

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SUPPORTING INFORMATION

Additional information may be found online in the Supporting Information section at the end of the article.

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