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ORIGINAL ARTICLE

Early onset of aging phenotype in vascular repair by Mas receptor deficiency

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Abstract Aging is associated with impaired vascular repair following ischemic insult, largely due to reparative dysfunctions of progenitor cells. Activation of Mas receptor (MasR) was shown to reverse aging-associated vasoreparative dysfunction. This study tested the impact of MasR-deficiency on mobilization and vasoreparative functions with aging. Wild type (WT) or MasR-deficient mice ($MasR^{-/-}$ or $MasR^{+/-}$) at 12–14 weeks (young) or middle age (11–12 months) (MA) were used in the study. Mobilization of lineage-negative, Sca-1-positive cKit-positive (LSK) cells in response to G-CSF or plerixafor was determined. Hindlimb ischemia (HLI) was induced by femoral artery ligation. Mobilization and blood flow recovery were monitored post-HLI. Radiation chimeras were made by lethal irradiation of WT or $MasR^{-/-}$ mice followed by administration of bone marrow cells from $MasR^{-/-}$ or WT mice, respectively. Nitric oxide (NO) generation by stromal-derived factor-1α (SDF) and mitochondrial reactive oxygen species (mitoROS) levels were determined by flow cytometry. Effect of A779 treatment on mobilization, blood flow recovery, and NO and ROS levels were determined in young WT and $MasR^{+/-}$ mice. Circulating LSK cells in basal or in response to plerixafor or G-CSF or in response to ischemic injury were lower in $MasR^{-/-}$ mice compared to the WT. Responses in $MasR^{+/-}$ mice were similar to the WT at young age but at the middle age, impairments were observed. Impaired mobilization to ischemia or G-CSF was rescued in WT → $MasR^{-/-}$ chimeras. NO levels were lower and mitoROS were higher in $MasR^{-/-}$ LSK cells compared to WT cells. A779 precipitated dysfunctions in young-$MasR^{+/-}$ mice similar to that observed in MA-$MasR^{+/-}$, and this accompanied decreased NO generation by SDF and enhanced mitoROS levels. This study shows that mice at MA do not exhibit vasoreparative dysfunction. Either partial or total loss of MasR precipitates advanced-aging phenotype likely due to lack of NO and oxidative stress.

Keywords Aging · Vasculogenic progenitor cells · Mas receptor · Ischemia

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Introduction

Bone marrow-resident hematopoietic stem/progenitor cells (HSPCs) have the potential to stimulate vascular regeneration and are mobilized into the circulation in circadian fashion [35]. HSPCs stimulate re-endothelialization of vasculature in the areas of ischemia and accomplish vascular regeneration largely by paracrine mechanisms [19, 75] therefore often termed as endothelial progenitor cells or vasculogenic progenitor cells (VPCs). Aging is associated

with increased risk for ischemic vascular disease characterized by vascular endothelial dysfunction and angiogenesis [56]. Accumulated evidence points to that the impaired regenerative functions of adult HSPCs are an underlying mechanism of increased risk for cardiovascular diseases with aging [28, 62]. Either decreased number of cells in the circulation or impaired regenerative potential of stem/progenitor cells was reported [24, 44, 55, 71]. G-CSF and plerixafor are clinically used for inducing mobilization of stem/progenitor cells from bone marrow into the blood stream and are currently used therapeutics for the collection of cells for autologous cell therapies [57]. Mobilization of VPCs by these agents was found to stimulate vascular regeneration in experimental models of ischemic injury and in patients with myocardial infarction [10, 23, 40, 64].

Despite strong evidence for vascular regeneration by bone marrow-derived VPCs in response to ischemic injury, evidence for transdifferentiation to endothelial cells is limited and variable. Hindlimb ischemia (HLI) model resulted in 0% of regenerated vasculature positive for bone marrow-derived cells [66, 76], when evaluated at 2–4 weeks post-HLI. In a mouse model of myocardial ischemia, < 3% of regenerated vasculature was found be engrafted with bone marrow-derived cells at 1–3 weeks following ischemic insult [16]. However, evidence for paracrine mechanisms was provided by many studies [3, 33, 63, 75]. Importantly, Ziebart et al. [75] showed that sustained presence of functional cells in the circulation at the time of ischemic insult is critical for the complete recovery of blood flow and revascularization of tissue. Consistent with the paracrine hypothesis, progenitor cells derived from pathologic environment showed angiocrine dysfunction [2, 20, 54].

The cardiovascular protective axis of renin angiotensin system (RAS) consists of angiotensin converting enzyme-2 (ACE2), which produces angiotensin-(1–7) (Ang-(1–7)) from Ang II or Ang-(1–9), and Mas receptor (MasR) that mediates the biological effects of Ang-(1–7). This was known to be counter-regulatory to the canonical RAS, ACE/Ang II/AT1 receptor pathway, which is known to be upregulated in renal and cardiopulmonary disorders [52]. MasR was first identified as a proto-oncogene as it was shown to induce tumorigenic properties in NIH3T3 cells [74]. However, later studies ruled out the involvement of MasR in the tumorigenic response [47]. MasR gene encodes a 7-transmembrane domain-containing structure similar to GPCRs and remained orphan without a known ligand [77]. An elegant study by Santos et al. [51] provided direct evidence for the Mas receptor (MasR) as a cognate receptor for Ang-(1–7) by determining binding affinity, and in vivo and in vitro functional studies in MasR$^{-/-}$ mice. This study has tremendously enhanced our understanding of the protective axis of RAS, ACE2/Ang-(1–7)/MasR. Genetic deficiency of MasR was shown to precipitate several cardiovascular, metabolic, and neurologic dysfunctions that are dependent on the genetic background. Cardiac fibrosis and hypertrophy were observed in MasR$^{-/-}$ mice in C57Bl/6 background [50]. Oxidative stress and endothelial dysfunction were reported in both C57Bl6 and FVBN background [46, 72]. Regional vascular resistance and hemodynamics are significantly altered in MasR$^{-/-}$ mice [8]. Metabolic changes included type 2 diabetes and dyslipidemia [53] with decreased PPARγ expression in adipocytes, and development of liver steatosis when fed with ApoE$^{-/-}$ mice [34, 59]. Importantly, genetic ablation of MasR abolished protection from high-fat diet-induced hypertension in females [69].

Recent studies provided strong evidence for vasoprotective functions of ACE2/Ang-(1–7)/MasR in the adult stem/progenitor cells [21]. Mobilization of VPCs was shown to stimulate vascular regeneration in experimental models of ischemic injury. ACE2 and MasR are expressed in human and murine VPCs [15, 17]. Activation of MasR by Ang-(1–7) or NorLeu^{3}-Ang-(1–7) stimulates vascular repair-relevant functions in human and mouse vascular progenitor cells including migration, proliferation, nitric oxide (NO) generation, and integration into the vasculature following ischemic injury, and by decreasing oxidative stress [60]. Importantly, vasoreparative dysfunctions induced by pathological conditions such as diabetes, pulmonary arterial hypertension, or heart failure are reversed by MasR activation [11, 17, 58, 65]. Total loss of MasR decreased mobilization of VPCs from bone marrow and blood flow recovery following ischemic injury at a young age, suggesting that MasR in the bone marrow is required for mobilization and vasoreparative functions nonetheless all mice were recovered from ischemic insult without amputations [65].

Recently, we have reported age-dependent decrease in ACE2 activity in the circulating VPCs in

human individuals that likely induces imbalance in local RAS resulting in decreased Ang-(1–7)/MasR activation and loss of vasoprotective functions in aging VPCs [24]. Consistent with this, in old mice of age 20–24 months, reparative functions of bone marrow-derived VPCs were impaired resulting in reduced blood flow recovery with toe or foot amputations following ischemic injury [24]. Importantly, reparative dysfunction was reversed by pharmacological activation of MasR by Ang-(1–7) [24]. The current study tested the hypothesis that partial or total loss of MasR-deficiency precipitates aging phenotype in vasoreparative functions. The study showed that mice do not show vasoreparative dysfunction at the middle age (MA) of 11–12 months; however, even a partial loss of MasR induced the dysfunction. The study was accomplished by determining mobilization of Lineage^{-}Sca-1^{+}cKit^{+} (LSK) cells from bone marrow into the circulation and blood flow recovery following ischemia in wild type (WT), MasR$^{+/-}$, and MasR$^{-/-}$ mice at young or middle ages. LSK cell population is enriched for hematopoietic progenitor cells with ~ 10% stem cells [45]. This population was consistently shown to be vasculogenic and accelerate vascular repair with a higher potential compared with other populations in experimental models of ischemic vascular diseases [13, 29]. Mobilization induced by clinically used mobilizing agents, plerixafor or granulocyte-colony stimulating factor (G-CSF), was evaluated. Radiation chimeras were used to determine if MasR expression in LSK cells or in the bone marrow microenvironment is required for mobilization and to test if repopulation of MasR$^{-/-}$ bone marrow with WT cells would reverse reparative dysfunctions. Besides, the impact of MasR-deficiency on intracellular levels of NO and reactive oxygen species (ROS) was evaluated. Lastly, studies were carried out to test if pharmacological blockade of MasR with A779 [49] precipitates early-aging phenotype of vasoreparative dysfunction in the young WT or MasR$^{+/-}$ mice.

Methods

Animal models

All animal studies were approved by the Institutional Animal Care and Use Committee at North Dakota State University (NDSU). Male or female wild type (WT), MasR$^{+/-}$, and MasR$^{-/-}$ mice in C57BL/6 background, at young age (12 to 14 weeks) or MA (11–12 months) [37], were used in the study. MasR$^{-/-}$ model was originally developed by KOMP repository and breeding pairs were kindly gifted by Prof. Cassis of the University of Kentucky. MasR$^{-/-}$ and the wild type controls and MasR$^{+/-}$ mice were bred and genotyped at the NDSU-animal facility. Mice were maintained on a 12-h light–dark cycle with food and water ad libitum.

Experimental procedures and treatment protocols

Mice were treated with plerixafor (AMD3100, Tocris) with a single dose of 5 mg/kg, s.c., or G-CSF (Peprotech) with a dose of 125 µg/kg/day, s.c., twice a day, for 4 days. Peripheral blood samples were collected with or without the treatment from submandibular vein under isoflurane anesthesia in citrate-containing tubes and were processed for flow cytometry and/or colony forming unit assay as described below. Mice were euthanized by thoracotomy under anesthesia followed by cardiac puncture, where applicable, for dissecting out femur and tibia. Whole bone marrow was obtained by crushing bones that were washed with not more than 1 mL of cold PBS. Suspensions were filtered and centrifuged to separate bone marrow from bone marrow supernatant. Supernatants were preserved at − 80 °C for further analysis. Where applicable, bone marrow pellets were resuspended in PBS and processed for isolation of a specific population of cells as described before. In selected experiments, mice were treated with A779 (Bachem) as a continuous perfusion subcutaneously by using osmotic pumps (Alzet) at the rate of 1 µg/kg/min for 4 weeks [65]. Mice were subjected to ischemic injury, as described below, in the week 4 of the treatment. Bone marrow-resident LSK cells from the treated groups were used for determining NO and mitoROS levels.

Flow cytometric analysis

Cells from either peripheral blood or bone marrow were prepared by lysing RBCs by suspending in a solution of 0.8% ammonium chloride and 2 mM EDTA. RBC-free cells were resuspended in cell staining buffer (BioLegend) for flow cytometry.

Trustain (BioLegend) was used for minimizing non-specific binding of antibodies. Then, cell suspension was incubated with fluorescent conjugated antibodies, Lineage cocktail-FITC, Sca-1-APC, c-Kit-PE (BioLegend). 7-AAD (BD Pharmingen) was added to detect dead cells prior to the flow cytometry. An aliquot of cells suspension with isotype controls was included in all experiments. Samples were analyzed for $Lin^{-}Sca\text{-}1^{+}c\text{-}Kit^{+}$ (LSK) cells in the monocyte-lymphocyte population by using a flow cytometer (Accuri C6) by using standard procedures that are applicable for the instrument. Dead cells, 7-AAD-positive, were excluded and doublets and clumps were excluded by using FSC-H vs FSC-L approach.

Radiation chimeras of WT and $MasR^{-/-}$ mice.

Chimeric mice were generated by lethal irradiation of mice followed by i.v. administration of one million bone marrow cells from the donor mouse. Lethal irradiation was carried out by exposing mice to 900 biorads of radiation in Cs-γ irradiator in two split doses at half-hour time interval located at the University of North Dakota (Grand Forks, ND). The following chimeras, WT → WT, WT → $MasR^{-/-}$, $MasR^{-/-}$ → WT, and $MasR^{-/-}$ → $MasR^{-/-}$, were obtained. Percent chimerism was determined by flow cytometric determination of LacZ-positive cells in the circulation as MasR gene was replaced by LacZ in $MasR^{-/-}$ mice. This was accomplished by using LacZ flow cytometry kit (ThermoFisher Scientific) that quantitating β-galactosidase activity by using fluorescein di-V-galactoside (FDG). Flow cytometry was performed as described above by using a flow cytometer (Accuri C6).

Hindlimb ischemia in mice

Under isoflurane anesthesia, femoral artery was ligated and excised at proximal and distal ends to induce hindlimb ischemia (HLI) in mice as described before [39]. Blood flow in the hindlimbs was measured by imaging the flux ($blood \times area^{-1} \times time^{-1}$) by using Laser Doppler imaging system (Moor Instruments Inc.) under isoflurane anesthesia, which was expressed relative to the mean blood flux in the contralateral non-ischemic limb. HLI-mobilized LSK cells were enumerated at different time points over a period of 21 days by flow cytometry as described above.

Isolation of cells from bone marrow

Lineage-negative (Lin^{-}) cells were isolated by negative selection by using immunomagnetic enrichment kit (Stemcell Technologies Inc.) as per manufacturer's instructions. RBC-lysed bone marrow single-cell suspension was incubated with antibodies for binding CD5, CD11b, CD19, CD45R, 7–4, Ly-6G/C (Gr-1), and TER119-expressing cells. Then, the cells were labeled by tetrameric antibody complexes that recognize biotin and dextran-coated magnetic particles and antibody bound cells were then separated by using EasySep™ magnet to obtain Lin^{-} cells. These cells were further process to obtain $Lin^{-}c\text{-}Kit^{+}$ cells by using positive immunomagnetic selection kit (Stemcell Technologies). The cell population enriched by this method has $Lin^{-}Sca\text{-}1^{+}c\text{-}Kit^{+}$ immunophenotype. Where applicable, LSK cells were plated in RPMI1640 (GE Healthcare) in U-bottom, 96-well plate at a low density of 2×10^{4} cells/150 μL per well, until they are used for proliferation or migration assay for less than 24 h following isolation.

Data analysis

Results are expressed as mean ± s.e.m. Number of experiments "*n*" indicates the number of mice used per each treatment group. Treatments were compared for significant difference by paired or unpaired "*t*"-test, as applicable. Some datasets were compared by two-way ANOVA mixed-effects model with Tukey's post-test for multiple comparisons, by using Prism software (version 8.12, GraphPad Software, Inc.). Experimental groups were considered significantly different if $p < 0.05$.

Results

Firstly, we have enumerated the circulating number of LSK cells in young (12–14 weeks) and MA (11–12 months) WT and MasR-deficient mice. In the young group, $MasR^{-/-}$ mice have lower number of cells in the circulation compared to WT or $MasR^{+/-}$ mice (Fig. 1A, B, and C) ($p < 0.05$, $n = 8$). At MA, WT mice displayed similar number of

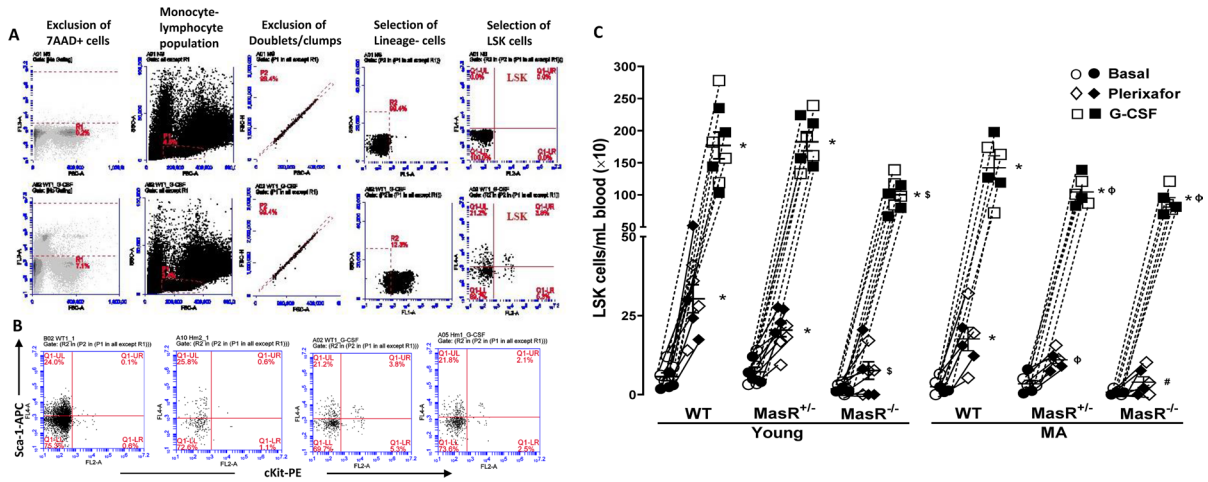


Fig. 1 Impaired mobilization of vasculogenic progenitor cells (LSK cells) by plerixafor or G-CSF in MasR-deficient mice at young (12–14 weeks) or MA (11–12 months) ages: **A** Schematic of flow cytometric determination of LSK cells in the mouse peripheral blood. Shown were representative flow cytometric dot plots in this process with isotype controls (upper panel) or with specific antibodies and 7AAD (lower panel). Events recorded in the upper right quadrant represent the number of LSK cells in that particular sample and were calculated per unit volume of blood. **B** Representative dot plots of circulating LSK cells on lineage-negative population in either wild type (WT) or MasR$^{-/-}$ mice before and after treatment with G-CSF. **C** Pair-wise comparison of responses to G-CSF or plerixafor in different experimental groups ($n=6$–8 per group). Open symbols—females and dark symbols—males. Each pair indicates pre- and post-treatment responses. *Compared to the respective basal levels ($p<0.01$ to 0.0001). $Compared to the young WT or MasR$^{+/-}$ ($p<0.05$ to 0.0001). ϕCompared to the young or MA WT and young MasR$^{+/-}$ ($p<0.05$ to 0.001). #Compared to the young or MA-WT and young or MA MasR$^{+/-}$ ($p<0.05$ to 0.0001)

circulating cells compared to the respective young. However significant decrease was observed in MasR$^{+/-}$ mice compared to the respective young. In MasR$^{-/-}$, circulating cells remained lower compared to the age-matched WT. Both males and females showed similar dysfunction.

Then, we tested mobilization of LSK cells in response to plerixafor or G-CSF. In the young group, a single dose of plerixafor (5 mg/kg, s.c.) increased the circulating LSK cells from 36 ± 12 to 310 ± 71 cells/mL blood in an hour in the WT mice (Fig. 1C). This response was decreased in MasR$^{-/-}$ mice. Plerixafor-induced mobilization was 76 ± 18 cells/mL blood ($p<0.005$ vs mobilization in WT, $n=8$) from the basal level of 7 ± 2 cell/mL blood ($p<0.03$ vs WT). This response in MasR$^{+/-}$ mice was similar to that observed in WT (Fig. 1C). In the MA group, plerixafor response was decreased in MasR$^{+/-}$ mice compared to age-matched WT ($p<0.01$, $n=6$). In the MA MasR$^{-/-}$, plerixafor response remained weaker compared to age-matched WT or young-MasR$^{-/-}$ ($p<0.01$, $n=6$). Along similar lines, G-CSF-induced mobilization was decreased in MasR$^{-/-}$ mice (930 ± 92 cells/mL blood) compared to the WT (1430 ± 52, $p<0.003$, $n=8$) (Fig. 1C) but not in MasR$^{+/-}$ mice in the young group. In the MA group, G-CSF response was not affected in WT compared to young or the age-matched, MA-WT while G-CSF response in MasR$^{-/-}$ group remained lower ($p<0.0001$ compared to young; $p<0.01$ compared to MA-WT). Two-way ANOVA detected significant difference in the responses to plerixafor or G-CSF between age groups ($p<0.001$) and among genotypes ($p<0.001$) with significant interaction ($p<0.0001$). Mobilization by either of the treatments was significant in all genotypes at both age groups compared to the respective basal levels (p ranging from 0.05 to 0.001) except the responses to plerixafor in the young MasR$^{-/-}$ or MA-MasR$^{+/-}$ or -MasR$^{-/-}$ mice, which were not significant (Fig. 1C).

In the next set of experiments, mobilization of bone marrow LSK cells into the blood stream in response to ischemic injury was determined. We have previously shown that young MasR$^{-/-}$ mice cannot mobilize cells from bone marrow in response to ischemic injury compared to the age-matched WT

[65]. In this study, we have explored this response in MasR$^{+/-}$ and MA groups. Mobilization response in young MasR$^{+/-}$ mice was similar to that observed in WT (Fig. 2A and B). Along similar lines, blood flow recovery following HLI is similar in WT and MasR mice in the young group (Fig. 2B). Both males and females showed similar responses to HLI. In the MA group, decrease in the mobilization was observed in WT (Fig. 3A and B), which was associated with decreased blood flow recovery compared to the young WT ($p<0.05$, $n=6$) (Fig. 3A and B). On the other hand, in both MasR$^{+/-}$ and MasR$^{-/-}$ groups, showed decreased blood flow recovery and experienced either toe or total foot amputations, 3/6 or 6/6 mice, respectively, by day 5 post-ischemic injury. Mice with amputations were removed from further studies in the experimental protocol.

Radiation chimeras were used to test if the impaired mobilization responses could be rescued by repopulating bone marrow in MasR$^{-/-}$ mice with that derived from WT mice. WT → MasR$^{-/-}$ chimeras showed 24 ± 1% LacZ^{+} cells suggesting 76% chimerism with WT cells ($n=6$) at 6–8 weeks following bone marrow transplants. While WT → WT chimeras, radiation controls, did not show any LacZ^{+} cells while MasR$^{-/-}$ mice have the maximum number (Fig. 4A). Transplantation with MasR$^{-/-}$ bone marrow could not rescue either WT or MasR$^{-/-}$ mice that have undergone lethal irradiation resulting in 100% mortality ($n=6$). Circulating LSK cells in WT → MasR$^{-/-}$ chimeras (38 ± 9) are comparable to that observed in WT → WT chimeras (40 ± 11) ($n=6$–8). G-CSF-induced mobilization was also similar in both groups of chimeras (612 ± 112 and 568 ± 97 cells/mL blood in WT → WT and WT → MasR$^{-/-}$ chimeras, respectively, $n=5$) (Fig. 4B). In contrast, plerixafor-induced mobilization was not observed in four out of six chimeras in both groups indicating the impact of γ-irradiation on the effects of plerixafor. In another set of experiments, ischemia-induced mobilization was tested in bone marrow chimeras. In WT → WT chimeras, radiation controls, LSK cells are mobilized in a significantly higher numbers on days 2 and 3 compared to pre-HLI with a peak mobilization response on day 2 (Fig. 4C). In WT → MasR$^{-/-}$ chimeras, mobilization of LSK cells was threefold on day 2 post-ischemia, which is similar to that observed in WT → WT chimeras (Fig. 4C). Along similar lines, no significant differences were observed in the blood flow recovery in WT → MasR$^{-/-}$ chimeras (84 ± 11%, $n=5$) compared to radiation controls (91 ± 11%, $n=5$) and no

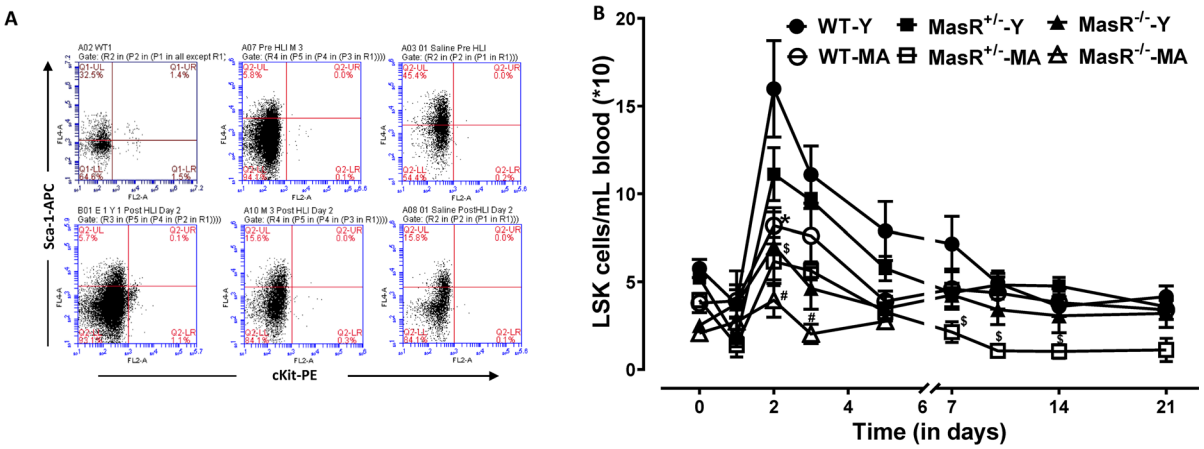


Fig. 2 Age-dependent impairment in the ischemia-induced mobilization of vasculogenic progenitor cells (LSK cells) in MasR-deficient mice: **A** Representative dot plots of circulating LSK cells before and after on lineage-negative population in either wild type (WT), MasR$^{-/-}$, or MasR$^{+/-}$ mice before or on day 2 following ischemic injury at young (Y) (12–14 weeks) or MA (11–12 months). **B** Summary of mobilization responses in young (C) or in MA (D) groups of wild type (WT), MasR$^{-/-}$, and MasR$^{+/-}$ mice ($n=6$ per group) following ischemic injury. Two-way ANOVA mixed repeated model detected significant differences among genotypes ($p<0.01$), age groups ($p<0.01$) with interaction ($p<0.001$). *$p<0.01$ compared to WT-Y. $Compared to WT-Y or MasR$^{+/-}$-Y ($p<0.05$ to 0.001). #Compared to WT-Y or MasR$^{+/-}$-Y and WT-MA or MasR$^{+/-}$-MA ($p<0.05$ to 0.001)

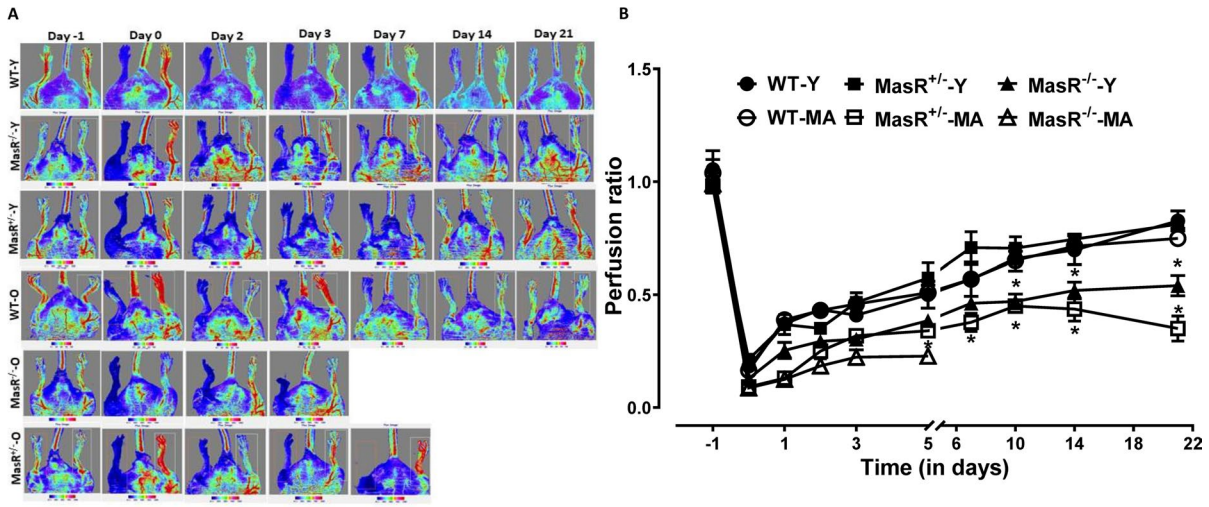


Fig. 3 Age-dependent impairment in the blood flow recovery following ischemic injury in MasR-deficient mice: **A** Representative pseudo-color images of blood flow in the hindlimb of mice following ischemic injury in either wild type (WT), MasR$^{-/-}$, or MasR$^{+/-}$ mice before and at selected time points following ischemic injury at young (12–14 weeks) or MA (11–12 months). **B** Summary of blood flow recovery expressed relative to that in the contralateral ischemic limb in young (**C**) or in MA (**D**) groups of wild type (WT), MasR$^{-/-}$, and MasR$^{+/-}$ mice ($n=6$ per group) following ischemic injury. MA MasR$^{+/-}$ and MasR$^{-/-}$ mice experienced amputations, 3/6 and 6/6 mice, respectively. Two-way ANOVA mixed repeated model detected significant differences among genotypes ($p<0.01$), age groups ($p<0.01$) with significant interaction ($p<0.01$). *$p<0.01$ compared to WT-Y, MasR$^{+/-}$-Y, or WT-MA ($p<0.05$ to 0.01)

amputations were observed. Thus, impaired mobilization and blood flow recovery to ischemic injury were restored by repopulating MasR$^{-/-}$ mice with WT bone marrow cells. It is important to note that γ-irradiation has significant impact on the mobilization response in WT mice as the response on day 3 was decreased in both chimeras. Based on these results the mobilization in different groups of mice is compared as follows:

WT HLI-2 = WT → WT = WT → MasR$^{-/-}$ > ($p<0.05$) MasR$^{-/-}$

WT HLI-3 > ($p<0.02$) WT → WT = WT → MasR$^{-/-}$ > ($p<0.05$) MasR$^{-/-}$

Then, we checked NO/ROS imbalance in MasR-deficient cells by flow cytometry. Basal NO levels were lower in MasR$^{-/-}$ LSK cells compared to WT cells ($n=6$) (Fig. 5A and B). Mean fluorescence intensity (MFI) of DAF-FM was increased by SDF (100 nM) or VEGF (30 nM) in WT cells indicating stimulation of NO generation, which was considerably reduced in MasR$^{-/-}$ cells ($p<0.01$ (SDF), $p<0.05$ (VEGF), $n=6$) (Fig. 5A and B). Next, we determined if NO depletion is due to the increased oxidative stress by determining cellular and mitochondrial ROS levels by using cell- and mitochondria-specific ROS-sensitive fluorescent dyes Cell-ROX and Mito-Sox by flow cytometry. MFI of both Cell-ROS and MitoSOX was higher in MasR-deficient cells compared to the WT cells ($p<0.05$, $n=5$) (Fig. 5C–F). To determine the impact of increased ROS on cell viability, mitochondrial membrane potential ($\Delta\Psi m$) and apoptosis were assessed in LSK cells by using Mitotracker-Red and Annexin V-flow cytometry. MFI of Mitotracker-Red was higher in MasR$^{-/-}$ LSK cells compared to the WT cells ($p<0.001$, $n=5$) (Fig. 5G and H). In agreement with this, the number of apoptotic cells were higher in MasR$^{-/-}$ LSK cells as indicated by increased MFI compared to WT cells ($p<0.003$, $n=5$) (Fig. 5G and H).

Lastly, in another set of experiments we tested if pharmacological blockade of MasR in young WT or MasR$^{+/-}$ mice would recapitulate the impaired vascular repair observed at MA. LSK mobilization and blood flow recovery following HLI were determined. Two-way ANOVA, mixed model with residuals, detected significant differences between genotypes ($p<0.01$) or treatments ($p<0.001$) with interaction ($p<0.001$). Treatment with A779 in the

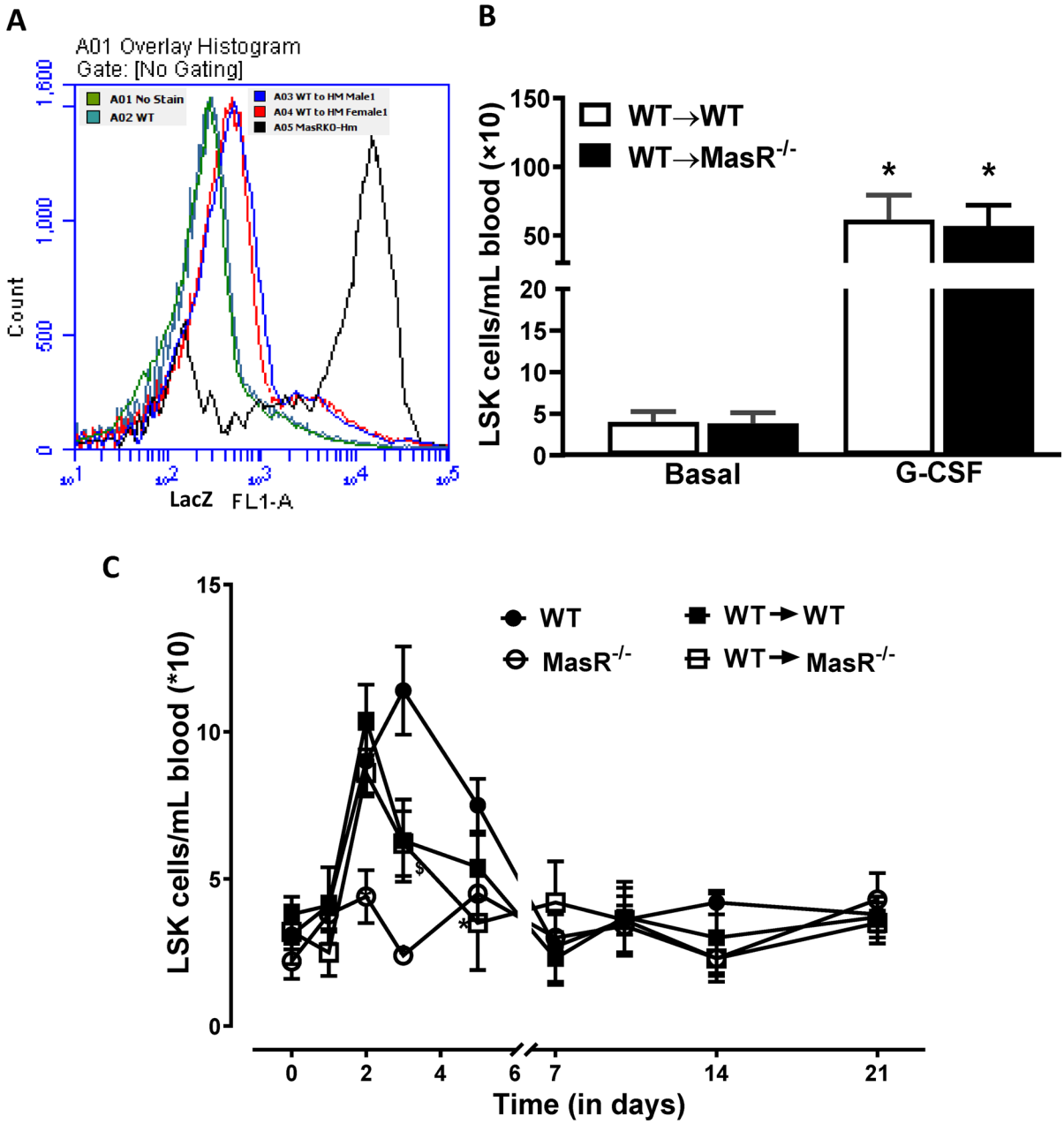


Fig. 4 Reconstitution of MasR$^{-/-}$ bone marrow with WT cells restores mobilization responses: **A** Representative flow cytometry histograms of LacZ^{+} cells to determine percent chimerism in WT→WT or WT→MasR$^{-/-}$ chimeras. Cells from WT and MasR$^{-/-}$ mice were included as negative and positive controls for LacZ^{+} cells. Left to right—no stain sample overlapping with WT→WT, two WT→MasR$^{-/-}$ chimeras, and MasR$^{-/-}$ mice. **B** Bar graph summarizing G-CSF-mobilization of LSK cells in WT→WT or WT→MasR$^{-/-}$ chimeras. No significant differences were observed in the two groups of chimeras wither in the basal or in response to G-CSF. *$p < 0.001$ compared to the respective basal levels. **C** Mobilization of LSK cells in WT, MasR$^{-/-}$, or chimeric mice before HLI (pre-HLI) or on day 1, day 2, or day 3 following HLI ($n = 5$). Two-way ANOVA detected significant difference among chimeras ($p < 0.05$) and time points ($p < 0.01$) following HLI with significant interaction ($p < 0.01$). *Compared to the respective time points in WT. $Compared to the respective time points in MasR$^{-/-}$ group ($p < 0.01$). #Compared to WT ($p < 0.05$)

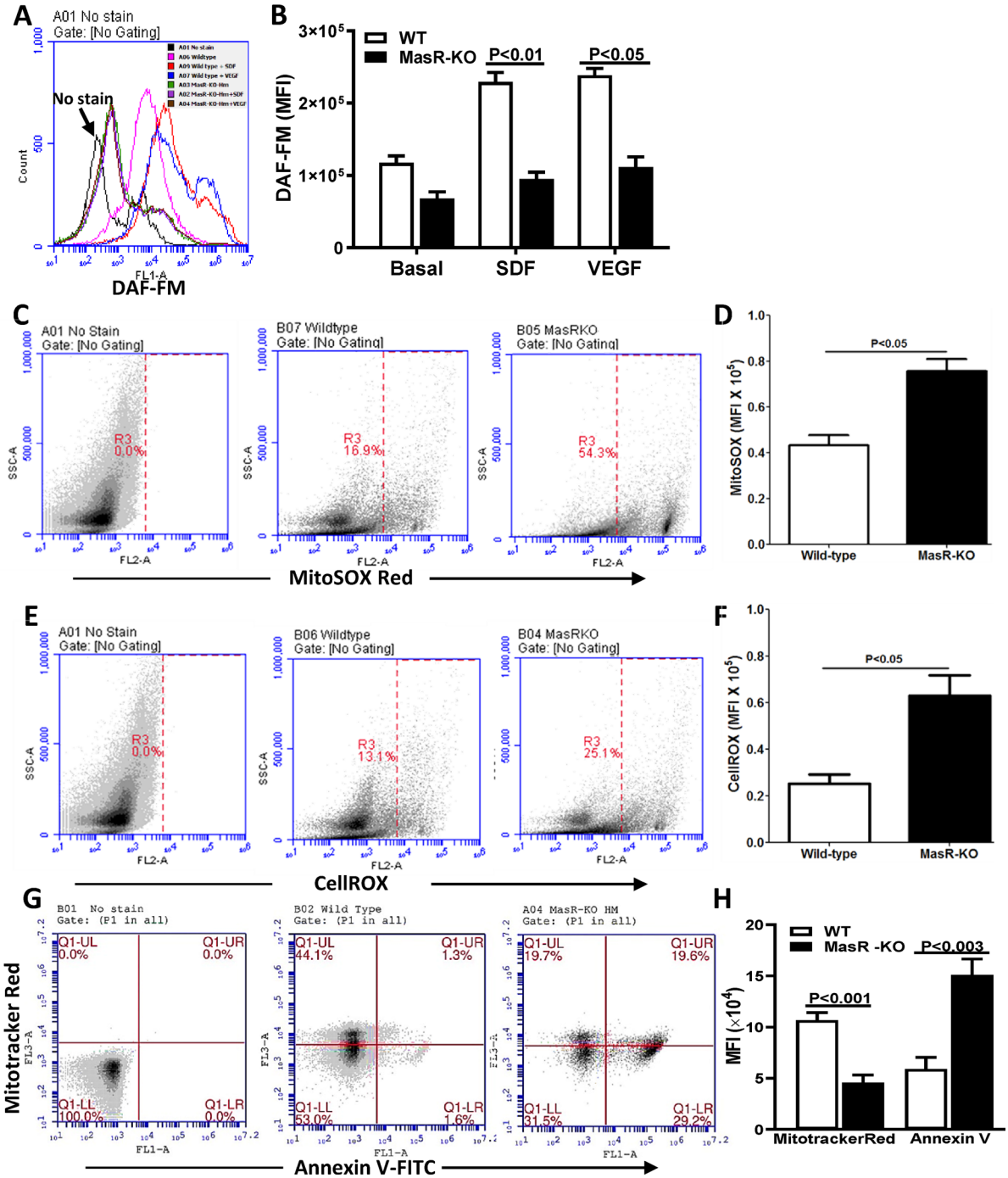
young-WT, significantly decreased LSK mobilization ($p<0.01$, $n=5$) (three males and two females) (Fig. 6A and B) and blood flow recovery ($p<0.001$) following ischemic injury. Maximum recovery of blood flow was $55\pm5\%$ compared to $92\pm5\%$ in the untreated group ($p<0.01$, $n=5$) (Fig. 6C and D). On the other hand, in the young-MasR$^{+/-}$ mice A779 treatment completely blocked LSK mobilization ($n=5$) (Fig. 6A and B). In agreement with the mobilization, decrease in the blood flow recovery was higher in A779-treated MasR$^{+/-}$ than that observed in the young-WT. Maximum recovery of blood flow was $32\pm7\%$ compared to $88\pm7\%$ in the untreated group ($p<0.01$, $n=5$) or treated WT ($p<0.05$, $n=5$) (Fig. 6C and D). Then, bone marrow LSK cells derived from all four treatment groups were evaluated for NO and mitoROS levels. SDF treatment increased DAF-FM fluorescence in cells derived from untreated WT or MasR$^{+/-}$ mice compared to the basal levels ($p<0.002$, $n=5$) (Fig. 7A and B). Nonspecific inhibitor of nitric oxide synthase (NOS), L-NAME, completely blocked SDF-induced increase in DAF-FM fluorescence in WT cells ($p<0.01$). In cells derived from A779-treated WT mice, SDF response was lower compared to that observed in cells from untreated WT ($p<0.05$, $n=5$). On the other hand, cells derived from A779-treated MasR$^{+/-}$ mice showed no significant increase in SDF-induced DAF-FM fluorescence compared to the basal levels. Along similar lines, MitoSOX fluorescence was similar in cells derived from WT or cells (Fig. 7C and D). In cells derived from A779-treated mice, MitoSOX fluorescence was higher compared to the untreated ($p<0.05$, $n=5$) and cells from MasR$^{+/-}$ mice showed higher MitoSOX fluorescence than the WT ($p<0.05$, $n=5$) (Fig. 7C and D). In the presence of Mito-Tempo, MitoSOX fluorescence was not observed in A779-treated MasR$^{+/-}$ cells.

Discussion

This study reports several novel findings that characterize the impact of total or partial loss of MasR on the vasoreparative potential with aging. Firstly, middle age of 11–12 months in mice is not associated with vasoreparative dysfunction. Partial loss of MasR is sufficient to induce vasoreparative dysfunctions in MA, mobilization of BM-resident VPCs, and blood flow recovery in response to ischemic insult, which increases risk for amputations. Secondly, mobilization of VPCs by G-CSF and AMD3100 is not impaired at middle age in mice; however, efficacy of either of the mobilizers is attenuated by partial loss of MasR. Importantly, the study demonstrated that MasR-deficient HSPCs cannot reconstitute bone marrow following radiation-induced depletion largely due to increased apoptosis due to mitochondrial oxidative stress and NO generation. Furthermore, repopulation of bone marrow in the MasR$^{-/-}$ with WT-HSPCs reversed reparative dysfunctions. Lastly, study showed that pharmacological blockade of MasR in young MasR$^{+/-}$ mice is sufficient to precipitate vasoreparative dysfunctions similar to that observed in MA-MasR$^{+/-}$ mice. While we have previously reported that total loss of MasR impairs vascular repair by VPCs [65], this study shows a partial deficiency would induce aging-like dysfunction [25] even at middle age in mice.

In the young group, total loss of MasR was found to be detrimental for complete recovery from an ischemic insult however no evidence for amputations of ischemic limbs. On the other hand, partial loss of MasR in MasR$^{-/+}$ mice had no impact on innate vascular repair following an ischemic injury. At an advanced age, the impact was severe in mice with either total or partial loss of receptors. We have previously showed that in mice at an age of ≥ 20 months, complete recovery from ischemic injury was impaired that resulted in foot partial amputations [24]. In the current study, mice of age 11–12 months have partially recovered blood flow following ischemic injury but with no amputations. Either a partial or total loss of MasR resulted in increased severity of ischemia following vascular injury and was associated with either toe or partial foot amputations. Thus, loss of MasR accelerated the occurrence of age-associated impairment in recovery from ischemic injury.

Functional vascular regeneration following ischemic injury is an orchestrated event involving bone marrow-derived progenitor cells and peri-ischemic endothelium [19]. In young mice, total loss of MasRs abolished mobilization of bone marrow-derived stem/progenitor cells into the circulation, which was not affected in mice with partial loss of MasR. At an advanced age, even a partial loss resulted in impaired mobilization. We have previously reported that at the age of ≥ 20 months, mobilization



was almost abolished in mice [24]. However, in the current study, at an age of 11–12 months, mobilization was significantly decreased, which was not observed in mice with partial or total loss of MasR. Thus, loss of MasR accelerated the occurrence of age-associated impairment in recovery from ischemic injury.

While there is no consensus in regards to the mechanism of G-CSF mobilization, G-CSF and AMD3100 act via distinct mechanisms. AMD3100

◀ **Fig. 5** Decreased NO generation and increased ROS levels in the MasR-deficient vasculogenic progenitor cells (LSK) cells: **A** and **B** Shown were representative histograms of DAF-FM flow cytometry in LSK cells from WT or MasR$^{-/-}$ cells either untreated or with different treatments with mean fluorescence intensity (MFI) and the number of events plotted on X- and Y-axes, respectively. Bar graph summarizing the effects of SDF or VEGF on NO generation in WT or MasR$^{-/-}$ cells. NO generation is decreased in MasR$^{-/-}$ cells in response to SDF ($p<0.01$, $n=5$) or VEGF ($p<0.05$, $n=5$). **C** and **D** Shown were representative dot plots of MitoSOX flow cytometry in LSK cells from WT or MasR$^{-/-}$ cells. MFI and the number of events plotted on X- and Y-axes, respectively. Bar graph summarizing mitochondrial ROS levels with MasR$^{-/-}$ cells showing higher intensity compared to WT ($p<0.05$, $n=5$). **E** and **F** Shown were representative dot plots of CellROX flow cytometry in LSK cells from WT or MasR$^{-/-}$ cells with MFI and the number of events plotted on X- and Y-axes, respectively. Bar graph summarizing cellular ROS levels with MasR$^{-/-}$ cells showing higher levels compared to WT cells ($p<0.05$, $n=5$). **G** and **H** Shown were representative dot plots of Mitotracker-Red and Annexin-V (FITC) flow cytometry in LSK cells from WT or MasR$^{-/-}$ cells with MFI and the number of events plotted on X- and Y-axes, respectively. Summary of ROS levels in WT or MasR$^{-/-}$ cells with MasR$^{-/-}$ cells showing higher intensity for both Mitotracker-Red ($p<0.001$) and Annexin V ($p<0.003$), compared to WT ($n=5$)

acts by blocking CXCR4 on stem/progenitor cells, which makes them nonresponsive to SDF resulting in disruption of the retention [9]. An alternative mechanism was proposed that involve activation of CXCR4 expressed on mesenchymal stromal cells resulting in secretion of SDF into peripheral blood thus reversing the retention gradient in favor of mobilization [12]. However, no explanation has been provided for the preferential binding to CXCR4 receptor on MSCs over other cell types including vasculogenic progenitors. On the other hand, several mechanisms were proposed explaining G-CSF mobilization. G-CSF increases proliferation and differentiation, and altering bone marrow architecture by increasing protease levels, leakage of blood vessels, osteoblast suppression, and partial disruption of VCAM/VLA4 as well as CXCR4/SDF interactions [6]. Despite the diverse mechanisms involved, mobilization by G-CSF or AMD3100 is attenuated by genetic ablation of MasR. At the MA, partial loss is sufficient to reduce the efficacy of these agents. In a previous study, we have reported that MasR-deficiency impacts SDF gradient in favor of retention of cells in the bone marrow [65],however, further studies are needed to understand the role of MasR signaling in mobilization and homing of bone marrow cells and involvement of different bone marrow niches.

Nitric oxide (NO) plays an important role in the mobilization and homing of vascular progenitor cells and genetic deletion or pharmacological inhibition of endothelial nitric oxide synthase (eNOS) interferes with mobilization [1]. NO levels are negatively modulated by reactive oxygen species (ROS) in the human VPCs [18]. MasR-deficiency was shown to impair vascular endothelial functions that was at least in part attributed to impaired NO generation and increased oxidative stress resulting in increased vascular reactivity and elevated blood pressure [31, 48]. In agreement with these findings, NO generation was impaired in response to hypoxia-regulated factor, SDF, in MasR$^{-/+}$ mice. In addition, mitochondrial ROS levels were increased in mice with MasR-deficiency, which would at least in part mediate depletion of NO availability. Previous studies have shown that MasR activation generates NO via PI3K/Akt pathway in human and murine cells [17, 70]. Oxidative environment, as in diabetes or obesity, depletes NO levels by direct scavenging or by eNOS uncoupling, which was reversed by MasR activation [38, 42]. Therefore, MasR activation stimulates NO levels in both physiological and pathological conditions and enhances NO-dependent vasoprotective functions. NO directly influences the migratory functions of vascular progenitor cells in response to hypoxia-regulated factors, SDF and VEGF, which is further corroborated by in vivo studies showing the requirement of functional eNOS/NO-cGMP signaling for physiological and ischemia-induced mobilization [1]. We have previously reported that migratory responses to SDF or VEGF are attenuated in the MasR-deficient vascular progenitor cells in vitro [65]. This dysfunction is very likely due to NO-deficiency and increased oxidative stress that impair migratory functions of vascular progenitor cells [18]. Physiological NO levels are critical for mobilization induced by plerixafor, and NO-deficiency impairs G-CSF mobilization, proliferation, and promotes apoptosis of cells [26, 41]. Optimal mitochondrial $\Delta\Psi m$ is required for ATP generation by ATP synthase; therefore, a balance in $\Delta\Psi m$ and intracellular ATP is pre-requisite for normal cell functioning [73]. Persistent decrease in mitochondrial $\Delta\Psi m$ is detrimental to the cell due to reduced ATP generation and mitochondrial stress [78], which would also explain increased apoptosis

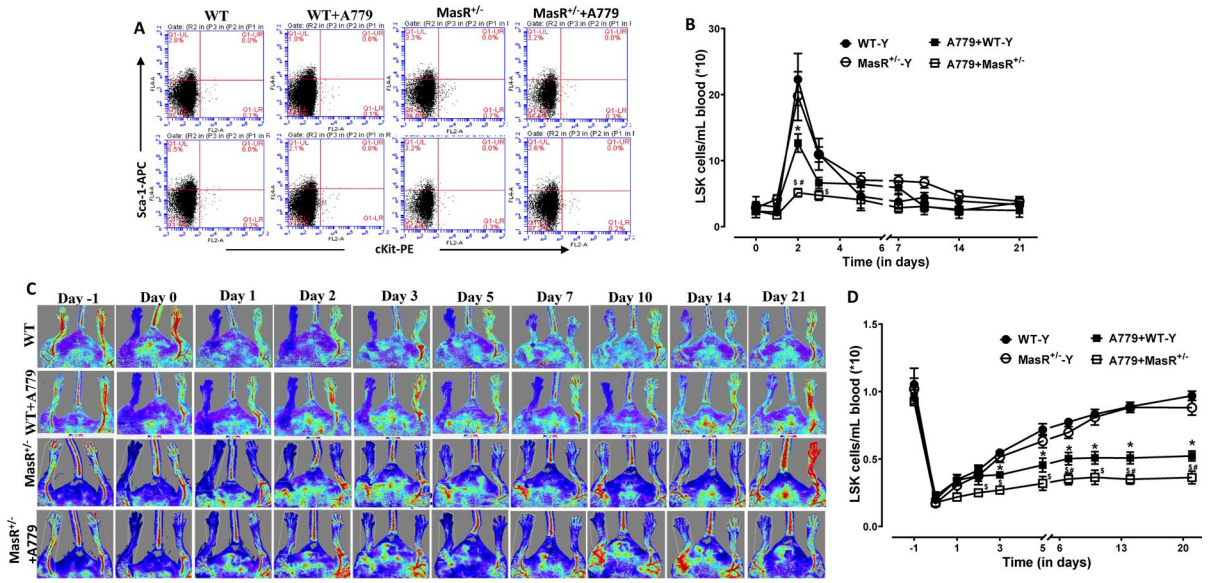


Fig. 6 Effect of A779 on LSK mobilization and blood flow recovery in young WT and MasR$^{+/-}$ mice: **A** Shown were representative flow cytometry dot plots of LSK cells from WT or MasR$^{-/-}$ mice treated with or without A779, before HLI (upper panel) and on day 2 post-HLI (bottom panel). **B** Summary of LSK mobilization responses in different treatment groups ($n=5$). Two-way ANOVA detected significant differences between genotypes ($p<0.01$) or treatments ($p<0.001$) with interaction ($p<0.001$). *$p<0.01$ compared with young-WT (WT-Y). \$$p<0.5$ to 0.001 compared with WT-Y or MasR$^{+/-}$-Y. #$p<0.01$ compared to A779-WT-Y group. Not indicated by an asterisk is A779 + MasR$^{+/-}$-Y compared to MasR$^{+/-}$-Y on day 10 ($p<0.05$). **C** Shown were pseudo-color Laser-Doppler images of perfusion in hindlimbs following ischemic injury in different treatment groups. **D** Summary of blood flow recovery response in different treatment groups ($n=5$). Two-way ANOVA detected differences between genotypes ($p<0.001$) or treatments ($p<0.001$) with interaction ($p<0.001$). *$p<0.05$ to 0.001 compared with WT-Y. \$$p<0.05$ to 0.001 compared with WT-Y or MasR$^{+/-}$-Y. #$p<0.05$ compared to A779-WT-Y group

in MasR-deficient cells. Effects of MasR activation or deficiency on mitochondrial function are unknown although angiotensin peptides and peptidases that generate Ang-(1–7) were identified in mitochondria derived from sheep kidneys (Wilson et al. 2016 AJP).

Studies with a pharmacological antagonist, A779, confirmed that the observed dysfunctions were indeed MasR-dependent. In young-WT mice, A779 significantly opposed mobilization and blood flow recovery but the effects were stronger in young-MasR$^{+/-}$ mice with complete blockade of mobilization and greater decrease in blood flow recovery as observed in MA-MasR$^{+/-}$ mice. A779 treatment also negatively impacted NO generation in response to SDF and increased mitoROS levels in young Mas$^{+/-}$ which was similar to that observed in MasR$^{-/-}$ mice. While dysfunctions induced by A779 are similar to that observed in MasR-deficient mice, severity of dysfunction in vivo was not totally similar with that observed in MA-MasR$^{+/-}$ mice as indicated by the lack of amputations. This can only be explained by chronic loss of the receptor, which would have a severe impact as opposed to the reversible receptor antagonism.

BM reconstitution involves homing of cells to BM niches, self-renewal, and differentiation to blood cells thus repopulating bone marrow with stem/progenitor cells. BM microenvironment is complex, consisting of microvessels, sinusoids, and many cell types including mesenchymal stromal cells (MSCs), osteoblasts, and CXCR12-abundant reticular (CAR) cells, and controls multiple functions of primitive HSCs [14]. Homing is a multistep process involving signaling by different cytokines and chemokines including SDF, adhesion molecules, and activation of proteolytic enzymes in an orchestrated pathway that causes rolling on endothelial cells followed by transendothelial migration to lodge in the BM [7, 30]. MasR$^{-/-}$ cells could not rescue WT mice following lethal irradiation indicating that MasR expression is required for

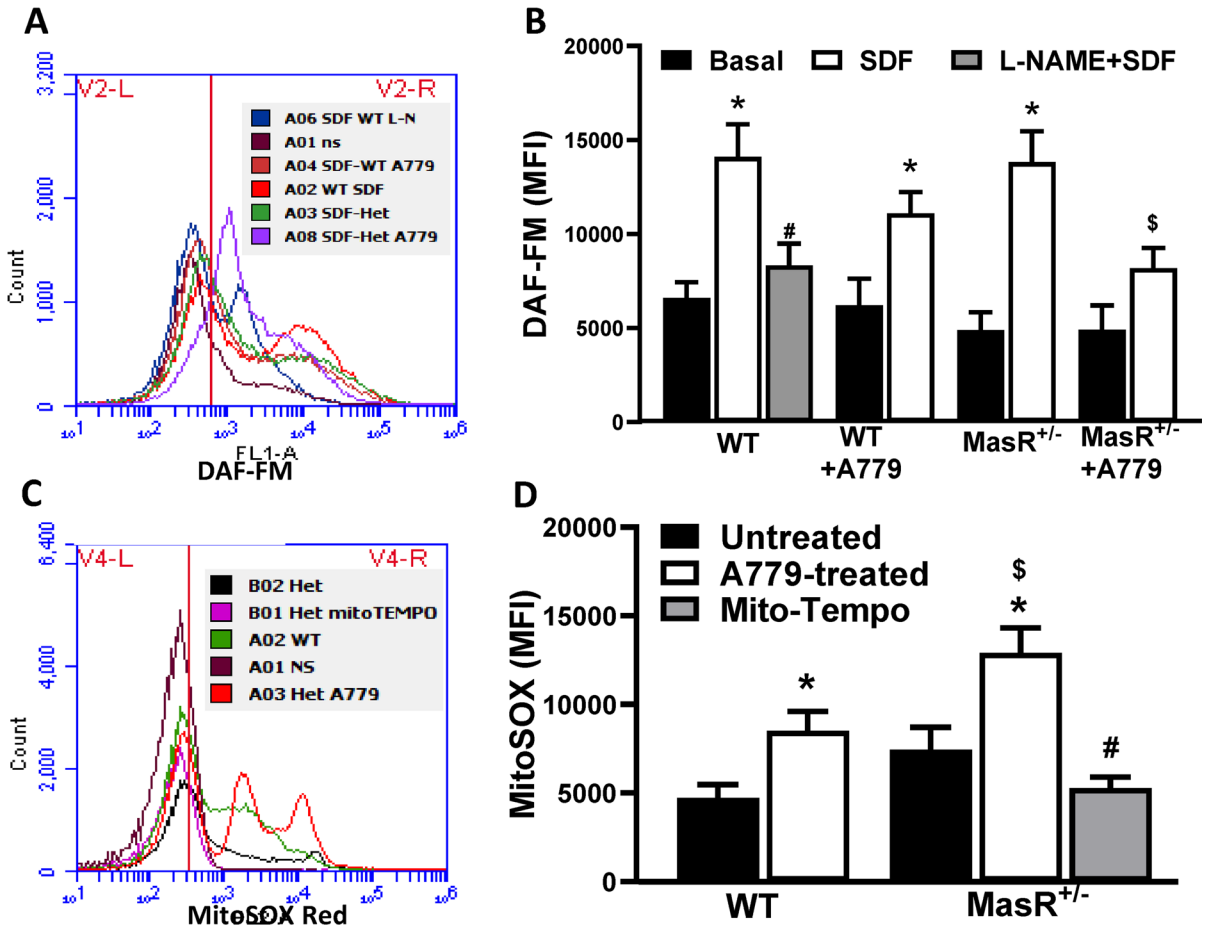


Fig. 7 Effect of A779 on NO generation and mitoROS levels LSK cells derived from young WT and MasR$^{+/-}$ mice: **A** and **C** Shown were representative flow cytometry histograms of DAF-FM and mitoSOX fluorescence, respectively, in LSK cells derived from WT or MasR$^{-/-}$ mice treated with or without A779. **B** Summary of the effect of A779 on NO generation by SDF in LSK cells derived from different treatment groups ($n=5$). *$p<0.05$ to 0.01 compared to the respective basal levels. #$p<0.05$ compared to SDF response in WT cells. \$$p<0.05$ compared to SDF response in MasR$^{+/-}$ cells. **D** Summary of the effect of A779 on mitoSOX levels in LSK cells derived from different treatment groups ($n=5$). *$p<0.05$ to 0.01 compared to the respective untreated cells. \$$p<0.05$ compared to mitoSOX fluorescence in cells from A779-treated WT. #$p<0.01$ compared to mitoSOX fluorescence in cells from A779-treated MasR$^{+/-}$

the homing and bone marrow engraftment of cells. SDF is necessary for direct homing of cells to the bone marrow and is NO-dependent as it is prevented in mice with genetic ablation of endothelial nitric oxide synthase (eNOS) or by L-NAME, a nonspecific NOS inhibitor [27]. Furthermore SDF-CXCR4 signaling activates transendothelial migration as well as adhesion of cells in LFA/ICA-1 and VLA4/VCAM1-dependent way [43]. While bone marrow SDF levels are not altered, lack of NO generation partly explains the impaired homing of MasR-deficient cells to WT bone marrow. In a limited number of experiments, we have observed decreased adhesion of MasR$^{-/-}$ cells to fibronectin in vitro (data not shown). As predicted, WT cells could home to and repopulate the bone marrow in MasR$^{-/-}$ mice suggesting that MasR$^{-/-}$ deficient microenvironment can support homing of cells. Furthermore, WT cells in MasR$^{-/-}$ mice responded to ischemic insult and mobilized and participated in revascularization of ischemic tissue. These findings collectively suggested that impaired vascular repair in MasR$^{-/-}$ is largely due to the dysfunction of VPCs and could be reversed by repopulating the bone marrow with functional WT cells.

Ang II generation and expression of AT1R increase with aging as demonstrated in vasculature derived from rats, primates, and humans [22, 67, 68]. In agreement with this, chronic suppression of Ang II generation by ACE inhibitors or the signaling by AT1R blockers ameliorates vascular inflammation and promotes longevity [4, 5, 36]. ACE2/Ang-(1–7)/MasR pathway is counter-regulatory to the ACE/Ang II/AT1R axis and is known to suppress expression of ACE or AT1R or AT1R signaling [52]. In agreement with this, MasR-deficiency or ACE2 blockade increased Ang II levels that in turn elevated the risk for cardiovascular detrimental effects such as aneurysm, atherosclerosis, or fibrosis [32, 61]. Therefore, authors speculate that the vasoreparative impairment and paracrine pro-inflammatory potential of LSK cells in MasR-deficient mice could be at least in part due to elevated Ang II signaling, which requires further investigation.

This study however has some limitations. Study did not attempt to localize bone marrow-derived cells in the ischemic areas following recovery as the vascular regeneration was largely attributed to angiocrine functions. At the same time, we could not determine pro-inflammatory factors that were known to be anti-angiogenic in the ischemic areas as muscle specimens could not be obtained in mice that had shown necrosis/gangrene or amputations. Quantification of NO and mitoROS levels in the bone marrow-derived cells rather than in the circulating cells, which would be more relevant however, adequate number of cells could not be isolated from peripheral blood for flow cytometry with all treatments for obtaining conclusive results. The study showed that irradiation of bone marrow impacted ischemia- as well as plerixafor-induced mobilization response in WT cells. Although comparisons were made between chimeras and radiation controls, study did not investigate the impact of radiation on specific bone marrow niches that would result in the observed deficiencies.

This study concludes that MasR is required for hematopoietic recovery and mobilization in response to ischemic injury and mobilizing drugs. Functional MasR is protective in preserving innate vasoreparative potential with advancement of age and that even a partial loss may precipitate dysfunctional phenotype of aging early in life. MasR signaling could be manipulated to enhance mobilization of stem/progenitor cells from bone marrow to blood stream in individuals that are characterized as poor mobilizers such as aging individuals for autologous cell therapies.

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Declarations

Conflict of interest The authors declare no competing interests.

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