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To link to this article DOI:
<http://dx.doi.org/10.1161/CIRCULATIONAHA.111.089763>

Publisher: American Heart Association

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Circulation. 2012;125:1774-1786; originally published online March 5, 2012;
doi: 10.1161/CIRCULATIONAHA.111.089763

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Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the
World Wide Web at:

<http://circ.ahajournals.org/content/125/14/1774>

Data Supplement (unedited) at:

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Role for Substance P–Based Nociceptive Signaling in Progenitor Cell Activation and Angiogenesis During Ischemia in Mice and in Human Subjects

Silvia Amadesi, PhD*; Carlotta Reni, MSc*; Rajesh Katare, MD; Marco Meloni, PhD; Atsuhiko Oikawa, PhD; Antonio P. Beltrami, MD, PhD; Elisa Avolio, MSc; Daniela Cesselli, MD, PhD; Orazio Fortunato, PhD; Gaia Spinetti, PhD; Raimondo Ascione, MD, ChM, FRCS, FETCS; Elisa Cangiano, MD; Marco Valgimigli, MD, PhD; Stephen P. Hunt, PhD, FMedSci; Costanza Emanuelli, PhD; Paolo Madeddu, MD

Background—Pain triggers a homeostatic alarm reaction to injury. It remains unknown, however, whether nociceptive signaling activated by ischemia is relevant for progenitor cells (PC) release from bone marrow. To this end, we investigated the role of the neuropeptide substance P (SP) and cognate neurokinin 1 (NK1) nociceptor in PC activation and angiogenesis during ischemia in mice and in human subjects.

Methods and Results—The mouse bone marrow contains sensory fibers and PC that express SP. Moreover, SP-induced migration provides enrichment for PC that express NK1 and promote reparative angiogenesis after transplantation in a mouse model of limb ischemia. Acute myocardial infarction and limb ischemia increase SP levels in peripheral blood, decrease SP levels in bone marrow, and stimulate the mobilization of NK1-expressing PC, with these effects being abrogated by systemic administration of the opioid receptor agonist morphine. Moreover, bone marrow reconstitution with NK1-knockout cells results in depressed PC mobilization, delayed blood flow recovery, and reduced neovascularization after ischemia. We next asked whether SP is instrumental to PC mobilization and homing in patients with ischemia. Human PC express NK1, and SP-induced migration provides enrichment for proangiogenic PC. Patients with acute myocardial infarction show high circulating levels of SP and NK1-positive cells that coexpress PC antigens, such as CD34, KDR, and CXCR4. Moreover, NK1-expressing PC are abundant in infarcted hearts but not in hearts that developed an infarct after transplantation.

Conclusions—Our data highlight the role of SP in reparative neovascularization. Nociceptive signaling may represent a novel target of regenerative medicine. (*Circulation*. 2012;125:1774-1786.)

Key Words: limb ischemia ■ myocardial infarction ■ neovascularization ■ stem cells

Trafficking of progenitor cells (PC) from bone marrow to the peripheral blood is tightly regulated by physical and paracrine interaction with stromal cells of the endosteal and vascular niches.¹ Tissue injury, such as acute myocardial infarction and limb ischemia, disrupts the retaining microenvironment, leading to forced PC egress into the circulation. Concurrently, the local release of cytokines, chemokines, growth factors, and neurohormones attracts circulating cells to the injury site.²⁻⁵ Pain is an essential component of the alarm reaction to tissue damage. However, the contribution of

nociceptive reflexes in PC mobilization during ischemia remains largely unexplored.

Clinical Perspective on p 1786

Sympathetic and primary afferent sensory fibers innervate the heart and peripheral tissues and are also expressed in bone and bone marrow (reviewed by Nance and Sanders⁶). After injury or thermal/chemical stimulation, sensory fibers release neuropeptides, such as substance P (SP) and calcitonin gene-related peptide (CGRP), from central terminals proj-

Received August 7, 2011; accepted February 27, 2012.

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The online-only Data Supplement is available with this article at <http://circ.ahajournals.org/lookup/suppl/doi:10.1161/CIRCULATIONAHA.111.089763/-/DC1>.

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Circulation is available at <http://circ.ahajournals.org>

DOI: 10.1161/CIRCULATIONAHA.111.089763

ecting to distinct brain stem levels, thus contributing to pain perception and pain-induced reactions. In addition, neuropeptides released from peripheral terminals of sensory neurons induce neurogenic inflammation, angiogenesis, and wound healing.^{2,7} Neuropeptides can also enter the systemic circulation, reaching distant organs where they regulate additional cellular responses. SP mediates its effects by preferentially binding and activating the tachykinin receptor neurokinin 1 (NK1), whereas CGRP acts on the calcitonin receptor-like receptor (CRLR), which is associated with and functionally regulated by the receptor activity-modifying protein 1 (RAMP-1).⁸

Growing evidence indicates the implication of bone marrow sympathetic fibers in the regulation of PC proliferation and mobilization.^{9–11} However, the role of nociceptors has not been investigated thoroughly.¹² A recent study showed that induction of corneal ulcers increases peripheral blood levels of SP, which in turn contributes to wound healing through the mobilization of mesenchymal stem cells from the bone marrow.² This seminal work suggests that noxious stimuli could activate cell mobilization through local nerves and neuropeptides from the circulation.

The present study investigates whether SP-based signaling modulates the mobilization of proangiogenic PC, thereby contributing to posts ischemic tissue healing. Our results newly show that ischemia induces reactive cellular responses in both animals and humans through the activation of SP release from peripheral nociceptors and modulation of SP content in bone marrow. This signaling mechanism is important for proper revascularization in animal models of ischemia.

Methods

Expanded methods are provided in the online-only Data Supplement.

Animal Studies

Seven- to 8-week old male CD1 and C57BL/6 mice (both from Harlan) and transgenic mice expressing an “enhanced” green fluorescent protein cDNA under the control of a chicken β -actin promoter and cytomegalovirus enhancer (Jackson Laboratory) were used. Furthermore, bone marrow reconstitution experiments were performed with the use of NK1-knockout (NK1-KO) or wild-type littermate mice as donors and sublethally irradiated wild-type mice as recipients. NK1-KO mice were generated by inserting a cassette containing an internal ribosome entry site and the *LacZ* coding sequence together with a neomycin resistance gene in exon 1 of the *NK1* gene.¹³

All experimental procedures were performed in accordance with the *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, 1996) and with approval of the British Home Office and the University of Bristol.

Myocardial Infarction Model

Myocardial infarction was induced by occlusion of the left anterior descending coronary artery.¹⁴ Twenty-four hours later, peripheral blood and bone marrow were collected for assessment of neuropeptide levels, immunohistochemistry, and flow cytometry analysis of cell antigenic profiles.

Limb Ischemia Model

Operative unilateral limb ischemia was induced as described previously.¹⁵ Mice were then allocated to specific experimental protocols (see below). Blood flow recovery was measured by laser Doppler flowmetry (Moor Instruments, UK) immediately after induction of ischemia and 3, 7, 14, and 21 days thereafter. Then mice were

ethanized, and the adductor muscles were collected for assessment of capillary and arteriole density.

Effect of Morphine on SP Release and PC Mobilization After Limb Ischemia

CD1 mice received morphine (20 mg/kg IP)¹⁶ 10 minutes before and 12 and 24 hours after induction of ischemia, whereas controls received vehicle. Peripheral blood and bone marrow were collected immediately before (time 0) and 1, 3, 12, 24, and 48 hours after induction of ischemia (n=3 per time point) for measurement of SP levels (EIA, Cayman Chemical) and flow cytometry assessment of NK1-expressing PC.

Effect of Bone Marrow Reconstitution With NK1-KO Cells on PC Mobilization and Reparative Angiogenesis

Wild-type mice were sublethally irradiated and then randomly assigned to receive 1×10^6 marrow cells from NK1-KO or wild-type mice through the tail vein (n=12 in each group). Eight weeks later, chimerism was verified on peripheral blood cells of recipient mice by assessing the expression of NK1 by polymerase chain reaction and *LacZ* transgene by β -galactosidase assay (Calbiochem, UK). Then mice were submitted to limb ischemia, and perfusion recovery was monitored until 21 days after ischemia. Peripheral blood samples were collected from the tail vein at day 3 to assess PC mobilization. At euthanasia, the adductor muscles were harvested after perfusion fixation for analysis of capillaries and arterioles. Cryosections of femurs were stained with a β -galactosidase antibody (AbD Serotec, UK) to confirm chimerism.

Effect of Exogenous SP on PC Mobilization

To demonstrate the direct effect of neuropeptide on mobilization, CD1 mice were injected with SP (5 nmol/L per kilogram IV; Bachem, UK),¹³ followed by collection of peripheral blood at 1, 3, 12, 24, and 48 hours (n=3 per time point) for measurement of SP and PC levels.

Transplantation of NK1-Enriched Cells in a Mouse Limb Ischemia Model

We next investigated whether the fraction of bone marrow cells that is functionally responsive to neuropeptides possesses proangiogenic activity in vivo. To this aim, bone marrow cells of enhanced green fluorescent protein–transgenic mice were submitted to a migration assay with the use of SP or CGRP as chemoattractant. The cells migrating to the lower chamber of the migration system (ie, SP- or CGRP-migrated cells [SP^{mig} and CGRP^{mig} cells, respectively]) or the cells migrating spontaneously in the presence of vehicle were harvested and, within 3 hours, transplanted into 3 different sites of the left adductor muscle of C57BL/6 mice at the occasion of limb ischemia induction (1×10^5 cells per 30 μ L phosphate-buffered saline per mouse). Control animals were injected with vehicle. Blood flow recovery was monitored until 21 days. At euthanasia, the adductor muscles were collected for analysis of neovascularization.

Immunostaining Procedures

Immunohistochemistry

Bones were fixed with 4% paraformaldehyde for 24 hours at 4°C and decalcified. Sections of 15- μ m thickness were mounted on poly-L-lysine-coated slides and processed for immunostaining.¹⁷ The immunosignal was amplified with the use of a tyramide signal amplification kit according to the manufacturer’s instructions (PerkinElmer).

The capillary and arteriolar densities of ischemic limb muscles were assessed with the use of isolectin B4 (Invitrogen) and α -smooth muscle actin (Sigma) staining as reported.¹⁵ Counts from 30 microscopic fields were averaged and expressed as the number of capillaries and arterioles per square-millimeter section.

Immunocytochemistry

Bone marrow cells were depleted from lineage-positive cells, and a cytospin from single-cell suspension was processed for immunostaining. Slides were mounted in Vectashield mounting medium containing 4',6-diamidino-2-phenylindole (DAPI) and observed with a confocal microscope with a $\times 63$ objective.

Cells and Cell Culture

Mouse Bone Marrow Cell Isolation

Bone marrow cells were depleted of mature hematopoietic cells by magnetic cell sorting with the use of a lineage cell depletion kit and a cocktail of lineage marker antibodies (MACS, Miltenyi Biotec) and then processed for immunocytochemistry or in vitro functional assays.¹⁸

Primary Culture of Mouse Sensory Neurons

Dorsal root ganglia (DRG) from mouse thoracic and lumbar spinal cords were cultured for 48 hours in Dulbecco's modified Eagle's medium containing 5% fetal bovine serum and 5% horse serum. 100 U/mL penicillin, 0.1 mg/mL streptomycin, and 2 mmol/L glutamine.¹⁹ DRG were then cultured overnight in 0.25% fetal bovine serum and 0.25% horse serum and next DRG and DRG conditioned media were used in migration assays.

In Vitro Assays

Migration

Cell migration was assessed with the use of transwell cell culture inserts with 3- to 5- μ m pore size filters as described.²⁰ Briefly, freshly isolated cells were plated in the upper compartment, and the test compound, DRG or conditioned medium was placed in the lower compartment. After 16 hours, cells from the upper (nonmigrated cells) and lower compartments (migrated cells) were collected and processed for flow cytometry analysis with the use of AccuCheck counting beads (Invitrogen) for absolute and reproducible quantification of cell numbers. Migration-induced enrichment of antigenically defined populations was expressed as the ratio of migrated to nonmigrated cells, followed by normalization to control (vehicle). This double normalization allows for direct control of changes in the antigenic profile that may have occurred during the migration assay. Furthermore, we know from pilot experiments that the attractant per se does not alter the cell antigenic characteristics. In selected experiments, cells were pretreated with CGRP and SP receptor antagonists for 30 minutes.

In Vitro Angiogenesis Assay

Migrated and nonmigrated cell fractions were cocultured on Matrigel with human umbilical vein endothelial cells for 16 hours at 37°C. Network formation was quantified by counting the number of branches per view field with the use of Image Pro-Plus software (Media Cybernetics). Each condition was performed with 6 biological replicates, and the assay was repeated 3 times. Counts of migrated cells were normalized by counts of respective nonmigrated cells.¹⁵

Flow Cytometry

Cells were stained with primary and secondary antibodies and then analyzed with the use of a FACS Canto II equipped with FACS Diva software (BD Biosciences).²⁰ In the text and figures, we will refer to cells expressing different markers by stating the cluster of differentiation marker (eg, CD117/c-Kit) followed by positive or negative in superscript format (eg, c-Kit⁺).

Western Blot Analysis

Protein extracts and immunoblot analyses were performed as described.²¹ Briefly, lineage-negative cells were starved for 3 hours in low serum medium and then treated with SP (100 nmol/L, 15 minutes in RPMI medium). Protein extracts from cell lysates were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and processed for Western blotting with the use of phospho-Akt

(Ser473) and total Akt antibodies (Cell Signaling Technology) and a horseradish peroxidase-conjugated anti-rabbit secondary antibody (Sigma).

Reagents

SP, CGRP, and α -CGRP₈₋₃₇, a CGRP receptor antagonist, were from Bachem. The NK1 antagonist RP67580 was from Tocris Bioscience. LY294002, a phosphoinositide-3 kinase antagonist, was from Calbiochem. Lineage markers and primary and secondary antibodies used for immunostaining are reported in Table I in the online-only Data Supplement.

Human Studies

Experiments on human samples complied with the principles stated in the Declaration of Helsinki and were covered by institutional ethical approval. Subjects gave written informed consent to sample collection.

Peripheral blood was obtained for assessment of neuropeptide receptor-expressing cells from patients with acute myocardial infarction and age-matched controls with similar risk factors but no evidence of coronary artery disease who participated in an observational clinical trial on the prognostic value of circulating PC at IRCCS MultiMedica, Milan, Italy (<http://www.clinicaltrials.gov>; identifier: NCT01271309) (Table II in the online-only Data Supplement). Moreover, migration assays followed by flow cytometry characterization of migrated cells were performed on peripheral blood mononuclear cells from 6 healthy subjects (average age, 30 years) and bone marrow mononuclear cells from 4 patients participating in the Bristol Heart Institute cell therapy trial TransACT1 (<http://www.controlled-trials.com>; identifier: ISRCTN65630838/TransACT) (Table III in the online-only Data Supplement).

Finally, human heart explants were collected from patients (n=9) who underwent cardiac transplantation 4 to 13 days after acute myocardial infarction at the University Hospital of Udine, Udine, Italy (Table IV in the online-only Data Supplement). Three of these patients had received a second transplantation for an infarct of the graft, which led in 2 cases to cardiogenic shock. Samples from infarcted area, border, and distant myocardium were obtained for immunohistochemistry of SP, NK1, CD34, and CD45 on 5- μ m-thick paraffin-embedded sections. Control specimens from comparable areas were sampled from explanted hearts that were judged not suitable for cardiac transplantation (n=5).

Statistical Analysis

Results are presented as mean \pm SEM. If data failed to pass normality and equal variance tests, a nonparametric analysis was applied, and results are expressed as median with 5 to 95 percentile distribution. Multiple groups were compared by parametric ANOVA, followed by Bonferroni *t* test, or nonparametric ANOVA on ranks, followed by Tukey pairwise comparison or Dunn test for multiple comparisons against a single control group. Analysis of the effect of bone marrow transplantation on postischemic blood flow recovery was performed with repeated-measures ANOVA followed by Bonferroni multiple comparison. Comparison of 2 groups was performed by paired or unpaired Student *t* test or Mann-Whitney rank sum test. $P < 0.05$ was considered significant. Stated n values represent biological replicates.

Results

Sensory Neuropeptidergic Neurons Are Present in Mouse Bone Marrow

Using the panneuronal marker Protein Gene Product 9.5 (PGP 9.5), we showed the presence of nerve fibers in the periosteum and endosteum and also in marrow perivascular areas of femur epiphysis (Figure I in the online-only Data Supplement). We also found that a subset of these fibers in trabecular bone and marrow is positive for nociceptor markers, such as SP (Figure 1), CGRP, and transient receptor

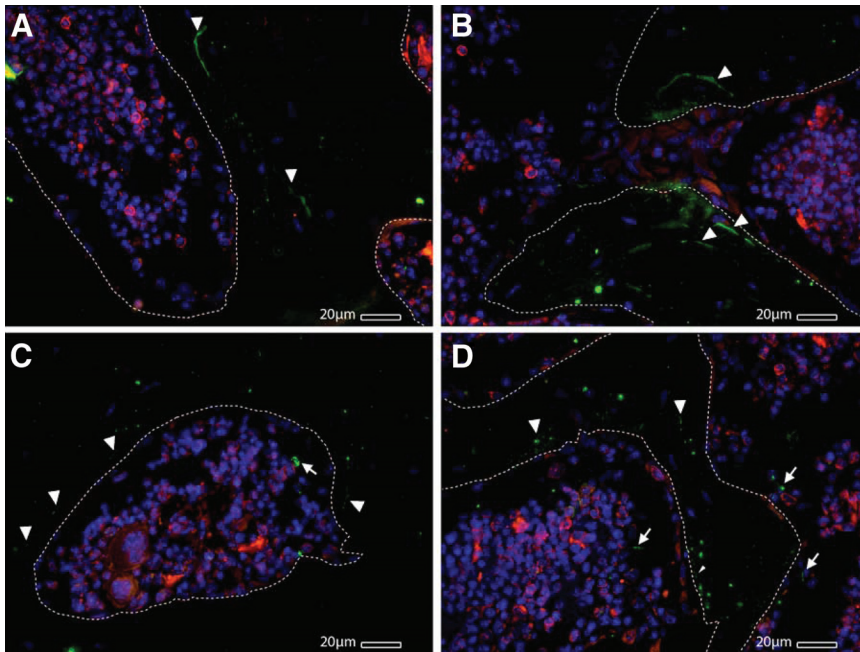


Figure 1. Trabecular bone and bone marrow contain nerve fibers that express SP. **A through D**, Representative fluorescence images of SP-positive nerve fibers (green fluorescence) traveling within both the bone spicules (**A through D**; arrowheads) and the marrow parenchyma (**C and D**; arrows). Bone edges are indicated by dashed lines, and hematopoietic cells are decorated by anti-CD45 antibody (red fluorescence). Nuclei are shown by the blue fluorescence of 4', 6-diamidino-2-phenylindole. Bars=20 μm .

potential cation channel subfamily V member 1 (TRPV1) (Figure I in the online-only Data Supplement).

We then analyzed the expression of SP and CGRP receptors on cytospin preparations of mouse bone marrow cells. We found that lineage-negative (Lin^-) cells express the SP receptor NK1 at the plasma membrane and also within cytoplasmic vesicles (Figure 2A). Similarly, we found that bone marrow cells express the CGRP receptors RAMP-1 and CRLR (Figure 2A through 2C). The immunoreactive signal was not detected when primary antibodies were omitted (Figure 2A and 2D). We further confirmed and quantified the percentage of cells expressing SP and CGRP receptors by flow cytometry. We found that freshly isolated bone marrow cells abundantly express NK1 ($86 \pm 1\%$), RAMP-1 ($50 \pm 2\%$), and CRLR ($77 \pm 2\%$) (Figure 2B through 2D). Moreover, neuropeptide receptor-positive cells coexpress markers for hematopoietic PC. Of the NK1⁺ cells (Figure 2B), $40 \pm 2\%$ were Lin^- , $28 \pm 2\%$ expressed c-Kit (the receptor for stem cell factor), and $8 \pm 1\%$ expressed Sca-1 (typical markers for hematopoietic PC). Of the RAMP-1⁺ (Figure 2C) and CRLR⁺ cells (Figure 2D), $45 \pm 2\%$ and $68 \pm 2\%$ were Lin^- , $22 \pm 1\%$ and $29 \pm 1\%$ expressed c-Kit, and $6 \pm 1\%$ and $5 \pm 1\%$ expressed Sca-1, respectively. Moreover, $15 \pm 3\%$ of the NK1⁺, $15 \pm 2\%$ of the RAMP-1⁺, and $52 \pm 9\%$ of CRLR⁺ cells also expressed CXCR4 (data not shown). Within the Lin^- Sca-1⁺c-Kit⁺ PC population, $98 \pm 1\%$ expressed NK1, $89 \pm 1\%$ RAMP-1, and $91 \pm 3\%$ CRLR. Nonhematopoietic cells identified as c-Kit⁺CD45⁻ cells also expressed NK1 ($62 \pm 4\%$), RAMP-1 ($65 \pm 7\%$), and CRLR ($73 \pm 7\%$) (data not shown). Thus, neuropeptides and their receptors are abundantly expressed in mouse bone marrow cells.

SP and CGRP Exert Chemoattractant Activity on Bone Marrow PC

We next assessed whether neuropeptides regulate bone marrow cells motility. Using the transwell migration assay, we

found that SP and CGRP (100–1000 nmol/L) exert a chemoattractant action on Lin^- cells (Figure 3A). These effects were reduced in cells treated 30 minutes in advance with the NK1 antagonist RP675880 (75% reduction of SP response; $n=3$; $P=0.005$) or the CGRP antagonist CGRP₈₋₃₇ (76% reduction of CGRP 100 nmol/L response; $n=4$; $P=0.001$). We also found that stimulation of Lin^- cells with SP for 15 minutes increases the phosphorylation of Akt, and this is prevented by the PI3K antagonist LY294002 (Figure 3B, top and left). Moreover, PI3K inhibition prevented PC migration induced by SP (Figure 3B, right). SP and CGRP (both at 100 nmol/L) increased cAMP production in Lin^- PC (SP, 1.5 ± 0.3 -fold; CGRP, 1.8 ± 0.5 -fold; $n=3$).

We next investigated whether neuropeptides released by nerve terminals exert a retaining action on bone marrow cells. To this aim, we used primary cultures of sensory neurons isolated from mouse DRG or conditioned medium in a migration assay on bone marrow cells. DRG and DRG conditioned medium induced the migration of bone marrow cells and the enrichment of c-Kit⁺Sca-1⁺ PC within the migrated fraction (Figure 3C and 3D). The chemoattractant effect induced by DRG was reduced by NK1 and CGRP antagonists (Figure 3E).

Ischemia Increases Peripheral Blood Levels of SP and Induces the Mobilization of NK1-Expressing Cells

Next we evaluated the mobilization of NK1- and CGRP-expressing cells in relation to changes of SP and CGRP levels in mouse models of acute myocardial infarction and limb ischemia. Circulating levels of SP were increased by 5-fold 24 hours after myocardial infarction compared with controls (236 ± 80 versus 48 ± 19 pg/mL, respectively; $n=6$ per group; $P<0.05$), whereas no difference between groups was detected in bone marrow levels of SP ($P=0.35$).

Analysis of distinct cell populations showed that myocardial infarction increases the abundance of granulocytes in

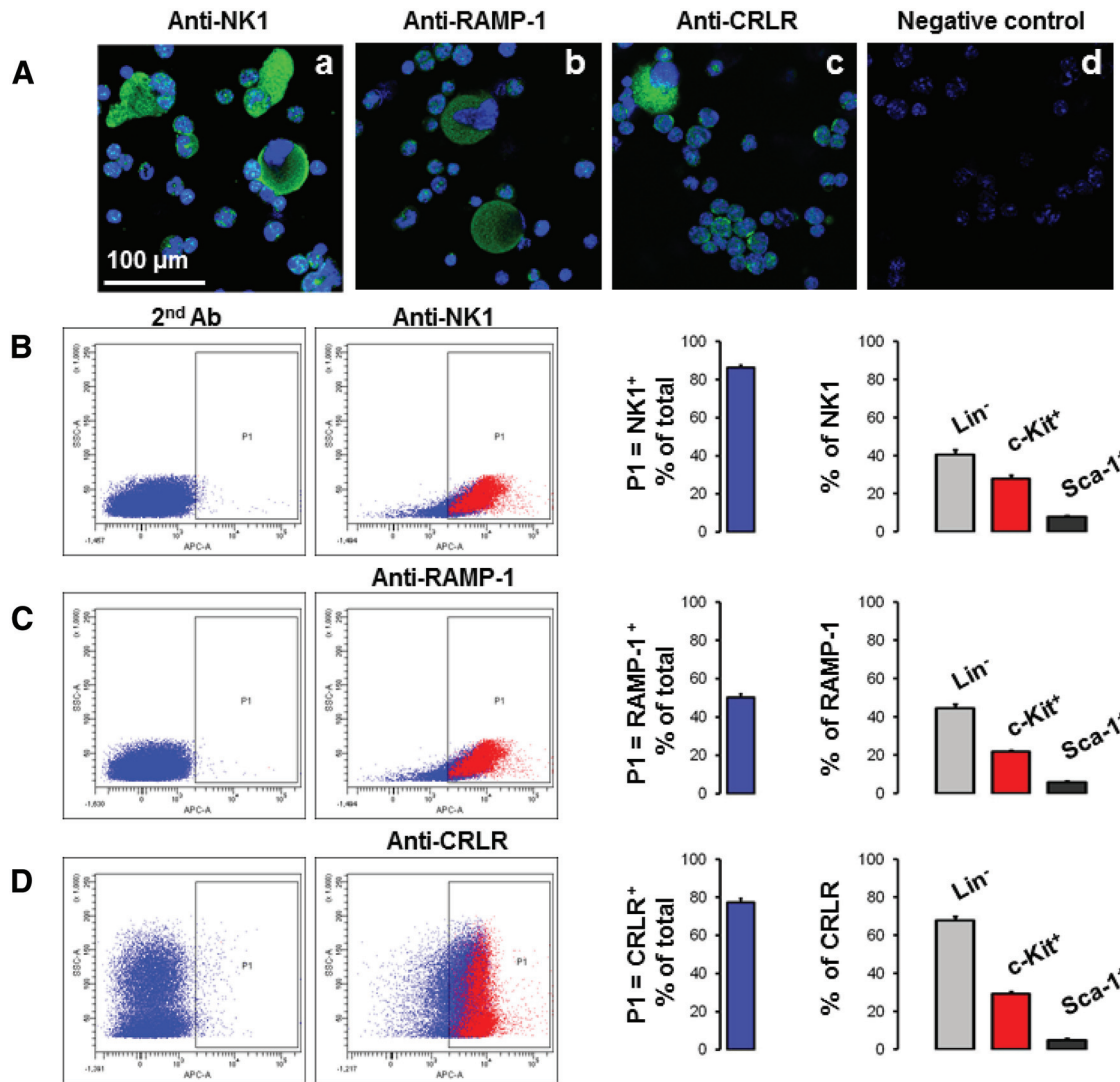


Figure 2. Bone marrow progenitor cells express substance P and calcitonin gene-related peptide receptors. **A**, Immunostaining of lineage-negative (Lin^{-}) cells (selected with the use of magnetic beads and a cocktail of antibodies against committed hematopoietic cells) expressing neurokinin 1 (NK1) (**a**), receptor activity-modifying protein 1 (RAMP-1) (**b**), and calcitonin receptor-like receptor (CRLR) (**c**) (green). Nuclei are stained with 4', 6-diamidino-2-phenylindole (blue). Immunoreactivity was not detected when primary antibodies were omitted (negative control, **d**). **B** through **D**, Flow cytometry confirms the expression of neuropeptide receptors in progenitor cells. Typical scatterplots and bar graphs show the analyzed data. A substantial fraction of $NK1^{+}$, $RAMP-1^{+}$, and $CRLR^{+}$ cells are Lin^{-} and express the progenitor cell markers c-Kit (c-Kit⁺) and Sca-1 (Sca-1⁺).

peripheral blood while reducing them in bone marrow (Figure II in the online-only Data Supplement). Similarly, myocardial infarction induced a 1.8-fold increase in $CD45^{+}c\text{-Kit}^{+}NK1^{+}$ granulocytes in peripheral blood ($12\,657 \pm 1532$ versus 6819 ± 827 cells per $100\ \mu\text{L}$ in controls; $n=6$ per group; $P<0.01$) and a reduction of their relative abundance in bone marrow ($41.0 \pm 0.6\%$ versus $52.0 \pm 1.0\%$ in controls; $P<0.01$). The abundance of total and $CD45^{+}c\text{-Kit}^{+}NK1^{+}$ lymphocytes/monocytes was not altered in both peripheral blood and bone marrow (data not shown). Moreover, myocardial infarction did not induce any change in circulating CGRP and in the abundance of cell populations expressing CGRP receptors (data not shown).

Limb ischemia caused opposite changes in the levels of SP in bone marrow (Figure 4A) and peripheral blood (Figure 4B), resulting in the modification of SP gradient between the

2 compartments. This was associated with an increased abundance of $Sca-1^{+}NK1^{+}$ (Figure 4F) and $Sca-1^{+}c\text{-Kit}^{+}NK1^{+}$ granulocytes (3-fold; data not shown) in peripheral blood from 24 hours after ischemia, whereas the change in $c\text{-Kit}^{+}NK1^{+}$ granulocytes was not significant (Figure 4E).

Nociceptive signaling is modulated by opioid receptors at the level of the central nervous system and primary afferent neurons. We next investigated whether opioid-induced analgesia interferes with the release of SP and the mobilization of nociceptor-expressing cells in mice with limb ischemia. Morphine inhibited the increase of SP in peripheral blood (Figure 4D) and blunted the decrease of SP in bone marrow (Figure 4C), thus nullifying the SP gradient between the 2 compartments. Moreover, we found that morphine remarkably attenuated the mobilization of $NK1$ -expressing PC after ischemia (Figure 4G and 4H).

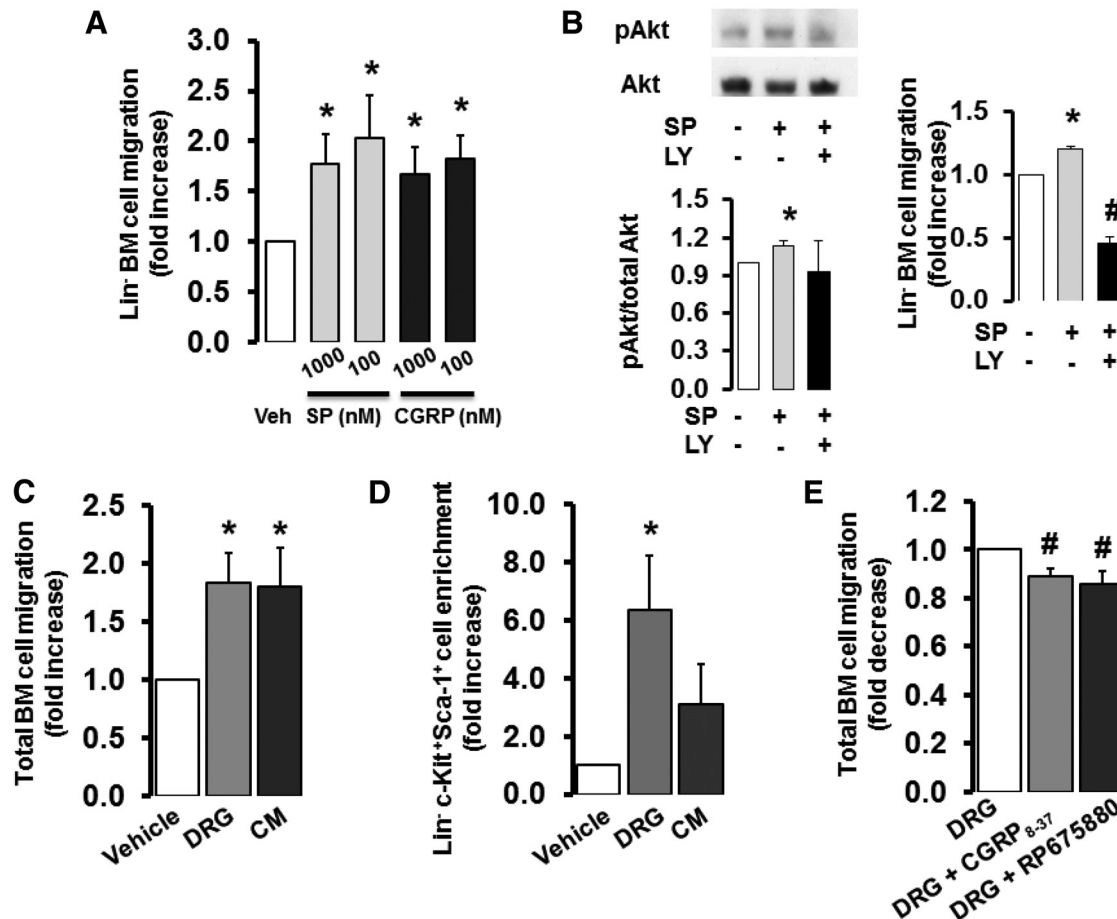


Figure 3. Substance P (SP) and calcitonin gene-related peptide (CGRP) exert chemoattractant effects on mouse bone marrow cells. **A**, SP and CGRP induce migration of lineage-negative (Lin⁻) bone marrow (BM) cells. Data are expressed as fold increase of vehicle (veh). **B**, SP (100 nmol/L for 15 minutes) induces phosphorylation/activation of Akt in Lin⁻ cells, which is prevented by the phosphoinositide-3 kinase antagonist LY 294002 (LY) (15 μ mol/L). * P <0.05 vs control; n =6 to 9. LY 294002 pretreatment also prevents SP-induced progenitor cell migration. * P <0.05 vs control; # P <0.05 vs SP only; n =3. **C** and **D**, Primary culture of mouse dorsal root ganglia (DRG) and respective conditioned medium (CM) induces cell migration (**C**) leading to an enrichment of Lin⁻ c-Kit⁺ Sca-1⁺ PC in the migrated fraction (**D**). **E**, The migratory effect induced by DRG is reduced by antagonists for CGRP (CGRP₈₋₃₇, 1000 nmol/L) and NK1 (RP67580, 100 nmol/L). * P <0.05 vs vehicle; n =6; # P <0.05 vs DRG alone; n =5.

In addition, we showed that systemic injection of SP per se, in the absence of ischemia, temporarily increases SP in peripheral blood and concomitantly induces the mobilization of Sca-1⁺ c-Kit⁺ NK1⁺ cells (Figure III in the online-only Data Supplement).

Role of NK1-Expressing Bone Marrow Cells in Postischemic Healing

To investigate the relevance of NK1-expressing cells in the reparative process after ischemia, sublethally irradiated mice were randomly assigned to bone marrow replacement with cells from NK1-KO or wild-type mice (n =12 in each group). Eight weeks later, mice were subjected to unilateral limb ischemia (Figure 5A). Mice transplanted with NK1-KO cells showed reduced peripheral blood levels of c-Kit⁺ granulocytes compared with controls transplanted with wild-type cells (Figure 5B). Moreover, repeated-measures ANOVA showed that blood flow recovery is delayed in the former group (Figure 5C). In agreement with this finding, NK1-KO cell recipients showed reduced reparative neovascularization at the capillary and arteriolar

levels compared with mice replaced with wild-type bone marrow cells (Figure 5D).

Neuropeptide-Induced Migration Enriches PC Able to Promote Reperfusion of Ischemic Limbs

To determine whether cells responsive to neuropeptide-induced chemoattraction are endowed with reparative activity, we transplanted mouse bone marrow cells that migrate toward SP (SP^{mig} cells) or CGRP (CGRP^{mig} cells) into the adductor muscle of mice with unilateral limb ischemia. Cells migrating toward vehicle (Veh^{mig} cells) were used as controls. Flow cytometry analysis of cells, before transplantation, indicated that migration itself provides enrichment for c-Kit⁺ PC, and this effect is enhanced when migration is stimulated by SP and CGRP (Figure 6A). Using laser Doppler flowmetry, we found that mice transplanted with SP^{mig} and CGRP^{mig} cells have improved blood flow recovery at 3 weeks after ischemia compared with mice injected with vehicle (no cells) or Veh^{mig} cells (Figure 6B and 6C). Analysis of ischemic adductor vascularization showed an effect of cell transplantation on arteriolar density in SP^{mig} and Veh^{mig} cell groups compared with vehicle (Figure 6D).

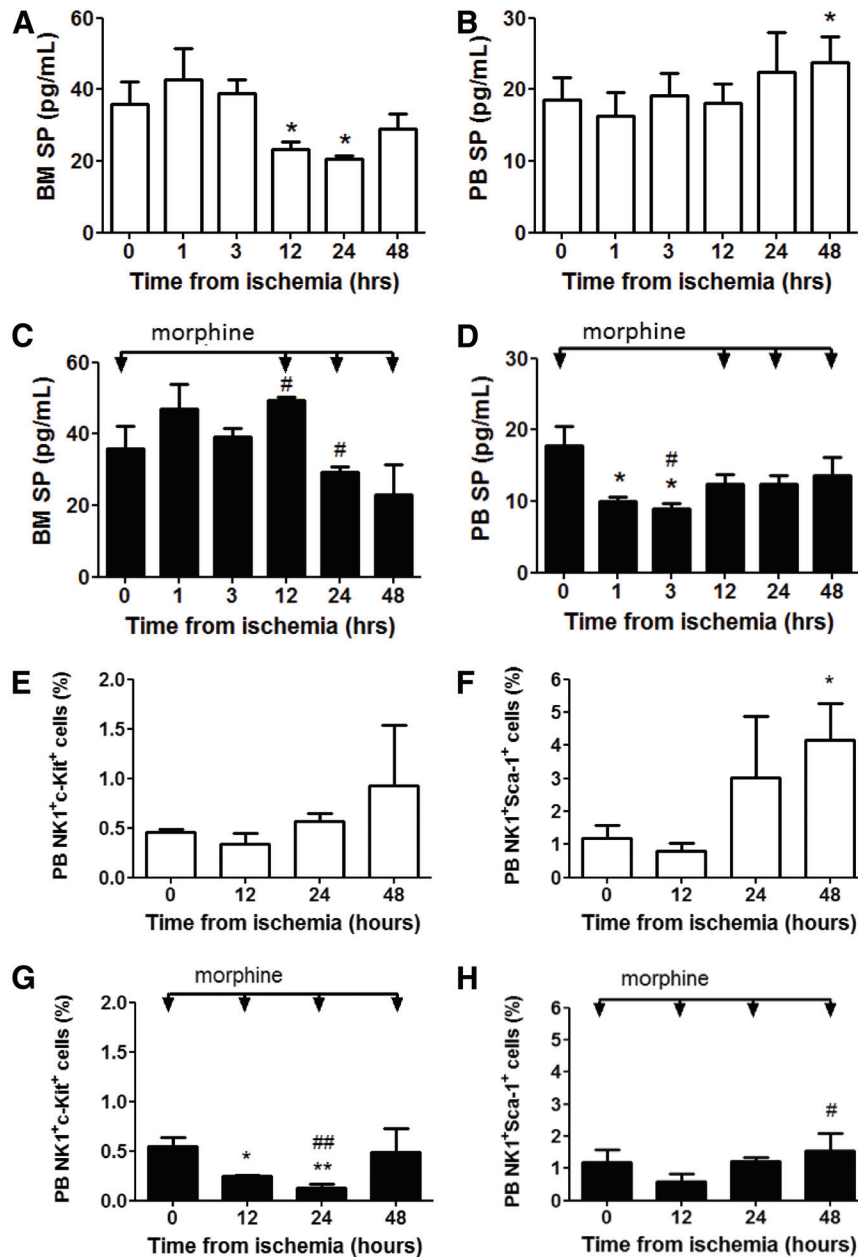


Figure 4. Limb ischemia induces progenitor cell mobilization, which is inhibited by morphine. **A** and **B**, Bar graphs show time course of changes in substance P (SP) levels in bone marrow (BM) (**A**) and peripheral blood (PB) (**B**) after induction of unilateral limb ischemia. **C** and **D**, Morphine blunts the SP decrease in bone marrow (**C**) and inhibits the SP increase in peripheral blood (**D**). **E** through **H**, Moreover, limb ischemia induces the release of PC expressing the neurokinin 1 (NK1) receptor (**E** and **F**), and this effect is inhibited by morphine (**G** and **H**). ANOVA followed by Dunnett multiple comparison test; * $P < 0.05$, ** $P < 0.01$ vs time 0; # $P < 0.05$, ## $P < 0.01$ vs corresponding time point in mice without morphine; $n = 6$ different mice per time point.

Human PC Express Neuropeptide Receptors

Circulating CD34⁺ PC from healthy subjects express neuropeptide receptors (Figure 7A) (gating strategies reported in Figure IV in the online-only Data Supplement) and migrate toward SP and CGRP (≈ 1.6 - and 1.2-fold increase compared with vehicle), and this migratory activity is inhibited by NK1 and CGRP antagonism (Figure 7B). Moreover, neuropeptide-induced migration provides enrichment for CD34⁺CXCR4⁺ (Figure 7C) and CD34⁺KDR⁺ PC (Figure 7D) coexpressing NK1 and RAMP-1. In particular, NK1 expression is increased in the CD34⁺CXCR4⁺ and CD34⁺KDR⁺ cell subfractions migrating toward SP. Likewise, CGRP induces a distinct enrichment of RAMP-1⁺ cells but does not enrich cells expressing CRLR. Interestingly, CD34⁺KDR⁺NK1⁺ and CD34⁺KDR⁺RAMP-1⁺ PC are attracted by both agonists. Furthermore, PC selected by SP- and CGRP-induced

migration are able to enhance human umbilical vein endothelial cell branch formation on Matrigel compared with nonmigrated cells, thus indicating the in vitro proangiogenic activity of cells that are functionally responsive to neuropeptides (Figure 7E).

We next asked whether human bone marrow cells also express neuropeptide receptors. Flow cytometry analysis of freshly collected bone marrow from patients with chronic myocardial ischemia showed the abundance of neuropeptide receptors on CD34⁺ cells, which are 75 \pm 15% NK1⁺, 70 \pm 20% RAMP-1⁺, and 75 \pm 20% CRLR⁺. Moreover, of CXCR4⁺ cells, 73 \pm 6% are NK1⁺, 55 \pm 15% RAMP-1⁺, and 56 \pm 22% CRLR⁺ ($n = 4$). We also found that SP and CGRP induce a migratory effect on human bone marrow mononuclear cells (1.3- and 1.2-fold increase, respectively, versus vehicle) (Figure V in the online-only Data Supplement).

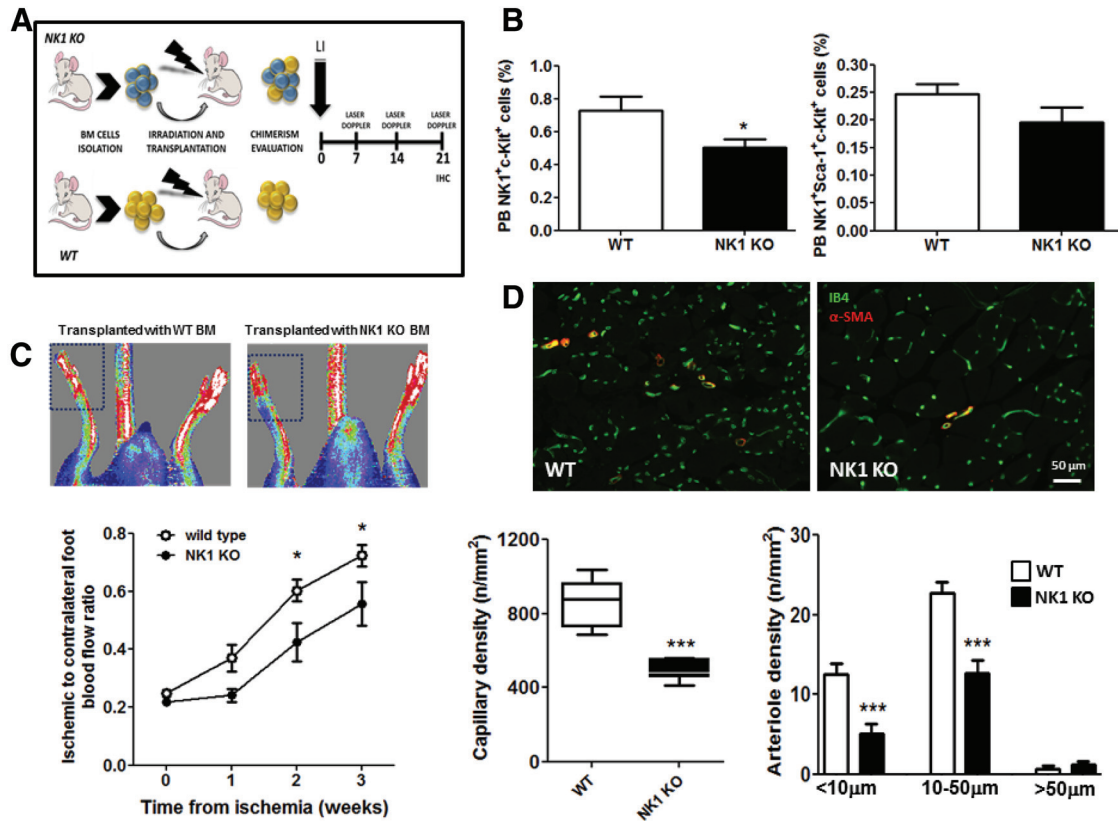


Figure 5. Bone marrow (BM) reconstitution with neurokinin 1 knockout (NK1-KO) cells inhibits reparative angiogenesis and perfusion recovery after limb ischemia. **A**, Schematic representation of the study protocol, consisting of BM reconstitution followed by induction of limb ischemia (LI). IHC indicates immunohistochemistry. **B**, Bar graph showing the levels of peripheral blood (PB) PC at 3 days after ischemia in mice that had their marrow reconstituted with wild-type (WT) or NK1-KO cells. **C**, Representative images of laser Doppler flowmetry captured at 3 weeks after induction of ischemia and line graph showing the time course of blood flow recovery in the 2 groups. **D**, Capillary and arteriole density in ischemic adductor muscles; endothelial cells are stained with lectin (green) and smooth muscle cells with α -smooth muscle actin (α -SMA) (red). Groups were compared by unpaired Student *t* test or Mann-Whitney rank sum test as appropriate. Analysis of blood flow recovery was performed by repeated-measures ANOVA followed by Bonferroni comparison; * $P < 0.05$, *** $P < 0.001$; $n = 9$ per group.

SP Modulates PC Mobilization and Homing in Patients With Acute Myocardial Infarction

Finally, we investigated the implication of the SP signaling pathway in PC mobilization and homing in patients with myocardial infarction. We found higher circulating SP levels in myocardial infarction patients (142 ± 32 pg/mL; $n = 23$) compared with healthy controls (46 ± 6 pg/mL; $n = 19$; $P = 0.001$) (Figure 8A), whereas CGRP did not differ between the 2 groups (30.5 ± 10.9 versus 14.7 ± 2.0 pg/mL; $P = 0.11$). This was associated with a remarkable increase in the relative abundance of cells expressing NK1 and RAMP-1 within the $CD34^+CXCR4^+$ and $CD34^+KDR^+$ PC fractions (Figure 8B and 8C) as well as in the proportion of $NK1^+CD34^+CXCR4^+$ and $NK1^+CD34^+CXCR4^+$ cells in total mononuclear cells ($0.010 \pm 0.003\%$ versus $0.003 \pm 0.001\%$ in controls; $P = 0.03$; $n = 10$ in both groups; and $0.006 \pm 0.001\%$ versus $0.004 \pm 0.001\%$ in controls; $P = 0.05$; $n = 10$ in both groups, respectively). The expression of CRLR was unchanged after acute myocardial infarction.

We next analyzed hearts of patients that were transplanted after acute myocardial infarction ($n = 6$). Ventricular fragments obtained from 5 explanted normal hearts that were judged not to be suitable for cardiac transplantation were

employed as controls. SP could be identified by immunohistochemistry particularly in the perivascular interstitium of the border zone (Figure 8D) and remote zone of infarcted hearts (Figure 8E) compared with controls (Figure 8F). Quantitatively, a significantly larger volume fraction of the region bordering the infarct was immunoreactive for this neurotransmitter (Figure 8G). We next investigated, on the same samples, the presence of $NK1^+$ cells expressing the endothelial and hematopoietic stem cell marker CD34. Moreover, CD45 was employed to label cells of clear hematopoietic origin. Both $CD45^+$ (Figure 8H) and $CD45^-$ cells (Figure 8I) coexpressing NK1 and CD34 were identified. Quantitatively, although $CD34^+NK1^+CD45^+$ cell density was significantly higher in myocardium distant from the myocardial injury, $CD34^+NK1^+CD45^-$ cells were significantly more frequent in the border zone compared with controls.

Finally, to verify whether denervated hearts had an impairment in the recruitment of $CD34^+NK1^+$ cells in response to injury, we compared hearts of patients undergoing cardiac transplantation after an acute infarct with hearts of patients retransplanted after an infarct of the graft ($n = 3$). In agreement with our hypothesis, both $CD34^+NK1^+CD45^+$ and $CD34^+NK1^+CD45^-$ cells were less abundant in this latter class of patients (Figure 8J).

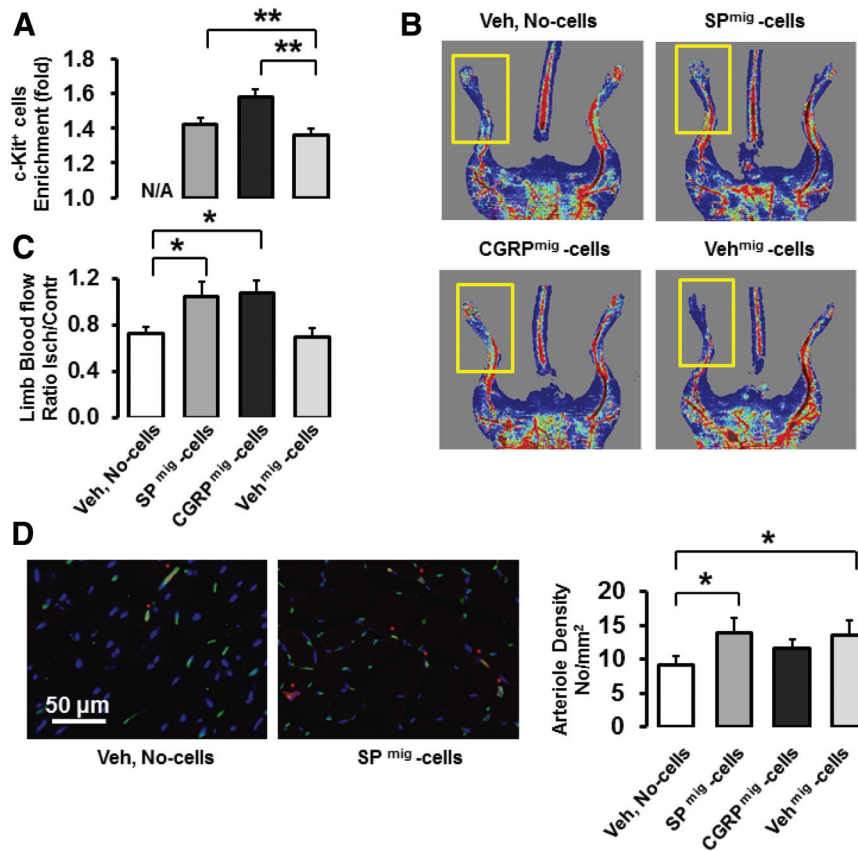


Figure 6. Substance P (SP)- and calcitonin gene-related peptide (CGRP)-enriched bone marrow progenitor cells stimulate the recovery of ischemic limbs. **A**, SP (SP^{mig} cells) and CGRP (CGRP^{mig} cells) induce migration and enrichment of progenitor cells that express the stem cell factor receptor c-Kit. Enrichment is expressed as ratio between c-Kit⁺ migrated and nonmigrated cells. **B** and **C**, SP and CGRP migrated cells improve the limb blood flow recovery at 3 weeks from ischemia induction compared with vehicle (veh). Original representative images (**B**) and average values (**C**) are shown. Isch indicates ischemia; contr, control. **D**, Histological analysis of angiogenesis with the use of immunostaining to visualize capillaries (isolectin B4; green) and arterioles (α -smooth muscle actin; red). Nuclei are stained with 4',6-diamidino-2-phenylindole (blue). Bar graph summarizes arteriole density data. ANOVA followed by Bonferroni comparison test; * $P < 0.05$, ** $P < 0.01$; $n = 8$ to 10.

Discussion

In this study, we identified the presence of primary nociceptive sensory fibers in bone marrow. We also found that bone marrow PC express NK1, the preferential receptor of SP, and migrate in response to SP stimulation. Ischemic injury remarkably increases SP levels in peripheral blood and induces the mobilization of proangiogenic PC, with these responses being abrogated by the opioid agonist morphine. Moreover, genetic disruption of the NK1 receptor in bone marrow cells results in defective PC mobilization, reduced reparative angiogenesis, and delayed postischemic recovery. NK1 receptor-expressing cells are abundant in human infarcted hearts but not in denervated hearts that suffered an infarct after transplantation. Hence, we conclude that the SP/NK1 duo is implicated in postischemic reparative response.

Primary Sensory Neurons Innervate Mouse Bone Marrow, and Bone Marrow PC Express SP and CGRP Receptors

The presence of sympathetic and sensory nerve fibers in bones and marrow of rodents has been reported previously.^{22–24} This study further identifies the intramedullary distribution of peptidergic primary sensory fibers, which were recognized by costaining for SP, CGRP, and TRPV1, a marker for capsaicin-sensitive fibers implicated in neurogenic inflammation and pain²⁵ (Figure I in the online-only Data Supplement). Multicolor fluorescence microscopy confirmed the distribution of SP-positive fibers in bone marrow parenchyma, thus providing an anatomic basis for the existence of neurogenic control of PC homeostasis (Figure 1). This concept is

strengthened by the other finding that bone marrow cells express neuropeptide receptors.^{26–29} Using flow cytometry, we newly document that NK1, CRLR, and RAMP-1 receptors are particularly abundant in mouse c-Kit⁺ PC (Figure 2) and human CD34⁺ PC (Figure V in the online-only Data Supplement). Both populations have been reported to participate in the postischemic regenerative process.^{4,30} Moreover, we report for the first time the ability of bone marrow cells to respond to neuropeptide stimulation in migration assays (Figures 3 and 7) as well as in vivo after intravenous administration of SP (Figure III in the online-only Data Supplement) or change in the SP gradient between bone marrow and peripheral blood after ischemia (Figures 4 and 8). Interestingly, PC expressing distinct neuropeptide receptors show competence to respond to both SP and CGRP, suggesting complementary/synergistic attraction by the 2 agonists. Moreover, because neuropeptide receptor-expressing cells coexpress chemokine receptors, such as CXCR4, neuroendocrine mechanisms may integrate the chemokine-mediated mechanism of PC mobilization and recruitment.³¹ The 2 mechanisms share common postreceptor signaling pathways. In fact, similar to SDF-1, which is the ligand of CXCR4, SP induces Akt phosphorylation in bone marrow PC. Inhibition of phosphoinositide-3 kinase contrasts the stimulatory effects of SP on Akt phosphorylation and PC migration.

Opioid Analgesia Abrogates the Mobilization of NK1-Expressing Cells After Ischemia

Pain is a typical symptom of acute ischemia and an essential component of the alert response to injury. Moreover, it is well known that opioid receptors and the SP receptor NK1 coexist

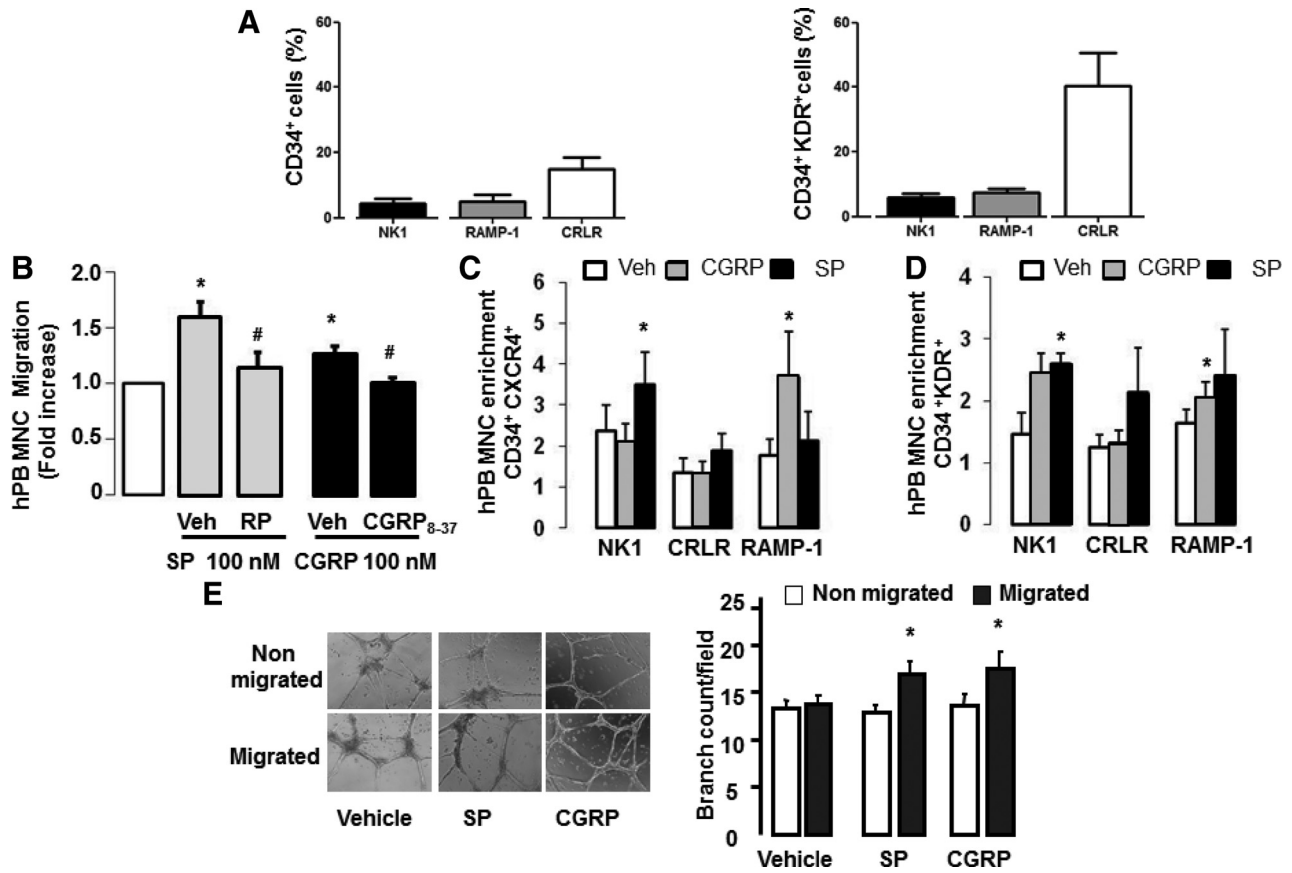


Figure 7. Neuropeptide receptors are expressed in circulating human progenitor cells. **A**, CD34⁺ progenitor cells from human peripheral blood (hPB) express neuropeptide receptors. NK1 indicates neurokinin 1; CRLR, calcitonin receptor-like receptor; and RAMP-1, receptor activity-modifying protein 1. **B**, Substance P (SP) and calcitonin gene-related peptide (CGRP) induce migration of hPB mononuclear cells (MNC), with this effect inhibited by antagonists for NK1 (RP67580, RP, 100 nmol/L) and CGRP (CGRP₈₋₃₇, 1000 nmol/L). **C** and **D**, Neuropeptides induce enrichment of hPB MNC expressing CD34/CXCR4 (**C**) and CD34/KDR (**D**) and coexpressing SP (NK1) and CGRP (RAMP-1) receptors. **E**, Circulating progenitor cells migrating toward SP and CGRP induce branch formation by human umbilical vein endothelial cells in a Matrigel angiogenesis assay. **P*<0.05 vs no treatment or vehicle; *n*=6. #*P*<0.05 vs SP or CGRP treatment plus vehicle; *n*=6.

and functionally interact in somatic and visceral sensory neurons, spinal cord projection and interneurons, midbrain, and cortex. Opioid receptors and neuropeptides like SP are synthesized in the DRG and transported along intra-axonal microtubules into central and peripheral processes of the primary afferent neuron. At the terminals, opioid receptors are incorporated into the neuronal membrane and become functional receptors. Activation of mature opioid receptors by endogenous ligands or systemically administered agonists potently inhibits SP release induced by peripheral noxious stimuli through coupling to G proteins that suppress cAMP-dependent Ca²⁺ or Na⁺ currents.³²

After acute injury, nociceptors and opioid receptors may also cooperate in the fine tuning of reparative responses. It was reported previously that morphine delays wound closure, reduces the number of circulating endothelial PC, and impairs reparative angiogenesis in mice.¹⁶ However, whether ischemic pain participates in mobilization of PC through the SP/NK1 duo has not been considered previously. We here report that acute myocardial infarction and limb ischemia increase the levels of circulating SP in mice and human patients, and this effect is associated with an augmented abundance of circulating NK1-expressing PC. Importantly, morphine abrogated the SP gradient

between bone marrow and peripheral blood and the mobilization of NK1⁺ PC (Figure 4), thus confirming the intertwined link between ischemic injury, SP signaling, and PC egress. However, further studies are warranted to address the role of pain afferents or efferents in this process.

The NK1 Receptor Is Fundamental for PC Mobilization and Reparative Response After Ischemia

Previous studies from Mishima et al³³ have shown that limb ischemia increases the expression of pro-CGRP mRNA and of CGRP protein in the lumbar DRG. In CGRP knockout mice, they observed impaired blood flow recovery from ischemia and decreased capillary density. Likewise, SP has been implicated in reparative angiogenesis, regulation of hematopoiesis, and recruitment of mesenchymal stem cells.^{2,7,34} To dissect reparative actions that could be ascribed to either local stimulation of angiogenesis or recruitment of proangiogenic cells by SP, we studied the postischemic recovery of mice whose bone marrow had been reconstituted with NK1-KO cells. Results indicate that the lack of NK1 receptor results in impaired PC mobilization, defective angiogenesis, and delayed perfusion recovery (Figure 5). There-

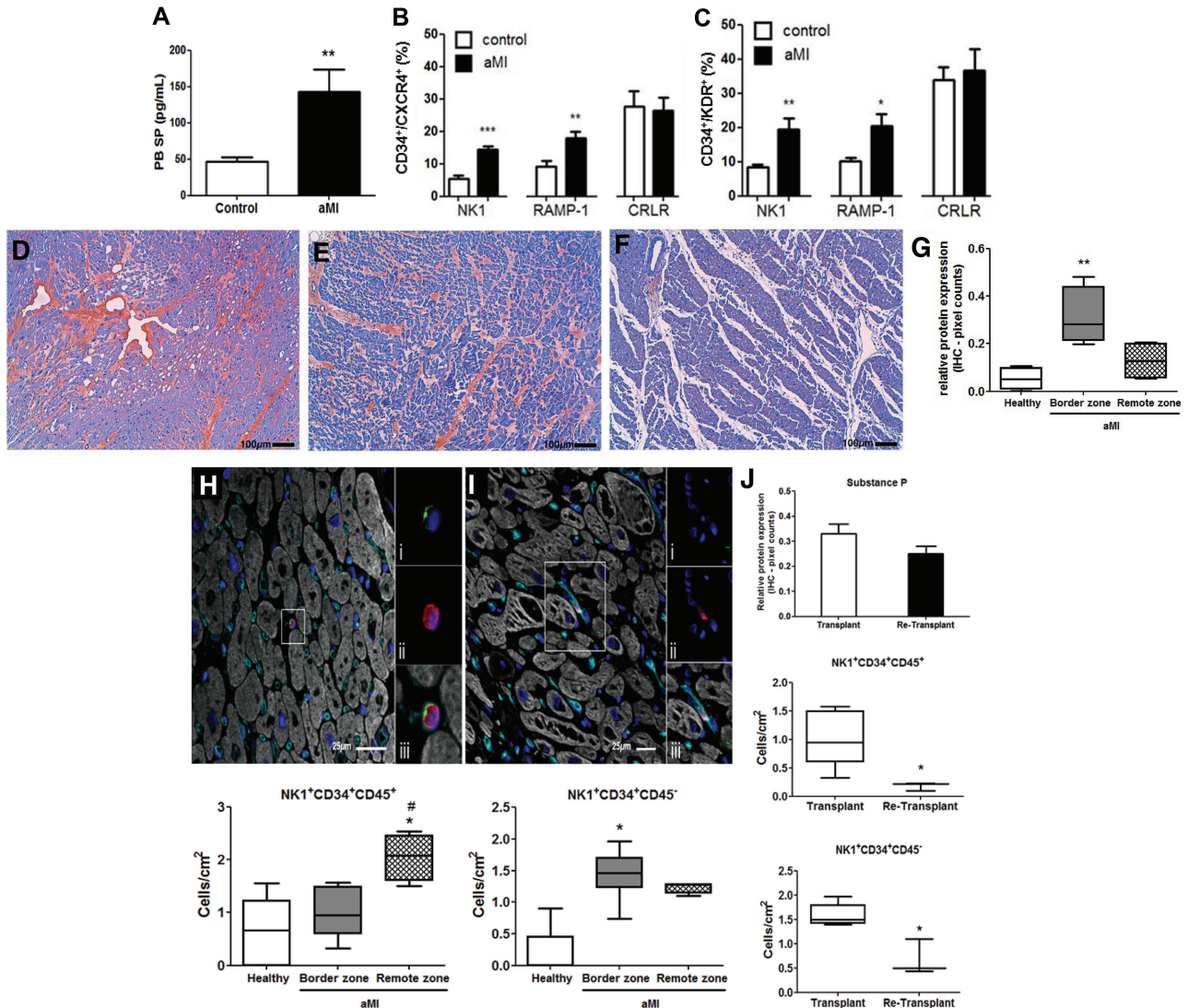


Figure 8. Activation of substance P (SP)-based nociceptive signaling after acute myocardial infarction (aMI) is associated with mobilization and homing of neurokinin 1 (NK1)-expressing progenitor cells. **A**, aMI increases peripheral blood (PB) levels of SP; $*P < 0.05$, aMI vs controls ($n = 23$ and 19 , respectively). **B** and **C**, aMI increases PB levels of PC positive for CD34/CXCR4 (**B**) and CD34/KDR (**C**) that coexpress NK1 and receptor activity-modifying protein 1 (RAMP-1) receptors. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ vs controls; $n = 10$ per group. CRLR indicates calcitonin receptor-like receptor. **D** through **F**, Representative microscopy images of SP staining in human myocardium at the level of infarct border zone (**D**) and remote zone (**E**). Normal myocardium shows low levels of SP expression (**F**). **G**, Bar graph showing the expression of SP in hearts of aMI patients ($n = 6$) and healthy controls ($n = 5$). IHC indicates immunohistochemistry. **H** and **I**, Abundance of NK1-expressing cells coexpressing CD34 and CD45 (**H**) or CD34 only (**I**). Bar graphs show the abundance of NK1⁺CD34⁺CD45⁺ cells (**H**) and NK1⁺CD34⁺CD45⁻ cells (**I**) in hearts of aMI patients ($n = 6$) and healthy controls ($n = 5$). $*P < 0.05$ vs healthy, $\#P < 0.05$ vs border zone. **J**, Levels of SP and abundance of NK1⁺CD34⁺CD45⁺ cells and NK1⁺CD34⁺CD45⁻ cells in hearts of transplanted ($n = 6$) and retransplanted aMI patients ($n = 3$). $*P < 0.05$ vs transplanted.

fore, an operative NK1 receptor is required for bone marrow cells to elicit reparative responses.

The spectrum of cells released after an ischemic event is heterogeneous and comprises inflammatory and regenerative subpopulations. We used a functional enrichment method based on *in vitro* migration to verify whether neuropeptide-responsive cells belong to the class of regenerative cells.²⁰ We found that human SP^{mig} and CGRP^{mig} cells enhance the formation of new branches of cocultured human umbilical vein endothelial cells (Figure 7). Moreover, mice transplanted with SP-responsive PC show increased arteriogenesis and improved blood flow recovery of the ischemic limb compared with vehicle-injected mice receiving injection of vehicle (Figure 6).

Homing of NK1-Expressing Cells in Infarcted Human Hearts

We report the presence of SP and NK1⁺CD34⁺CD45⁺ PC in the remote zone of infarcted human hearts (Figure 8). These cells are remarkably less abundant in posttransplant infarcted hearts, suggesting that denervation may have hampered PC recruitment. These data need to be confirmed in a larger series of transplanted hearts and in models of cardiac denervation. Moreover, we observed that NK1⁺CD34⁺CD45⁻ cells are more abundant in the border of infarcted human myocardium and mainly localized in vascular-like structures. It is not clear whether NK1⁺CD34⁺CD45⁻ cells are resident vascular cells or derive

from nonhematopoietic PC, which incorporate in peri-infarct neovascularization.

Clinical Impact

The present study identifies a novel regulatory mechanism triggered by ischemic injury and involving the release of SP from peripheral tissues into the circulation. This neural reflex also results in reduction of SP levels in bone marrow, at least after limb ischemia. The creation of a SP gradient between the 2 compartments facilitates the egress of NK1-expressing cells from bone marrow into the circulation, which is abrogated by opioid receptor stimulation. Moreover, disruption of NK1 on bone marrow cells jeopardizes reparative responses after ischemia.

These new findings may have important clinical implications. Dysfunction of the neurogenic mechanism triggered by ischemic injury may contribute to the impairment of post-ischemic repair during aging or degenerative diseases (eg, diabetes mellitus).^{35–37} Furthermore, pharmacological control of pain could be detrimental. In 1928, Sir James MacKenzie suggested treating cardiac patients with bed rest, morphine, and chloroform until unconsciousness ensued.³⁸ Eighty-three years later, the American College of Cardiology/American Heart Association guidelines continue to recommend intravenous morphine as a class IC indication for patients with suspected acute coronary syndromes whose pain is not relieved after nitroglycerin or whose symptoms recur.³⁹ However, results from the CRUSADE Quality Improvement Initiative showed that morphine is associated with higher mortality in patients with acute coronary syndrome after risk and treatment adjustment.⁴⁰ Whether this detrimental effect is attributable to suppression of nociceptor-mediated PC release remains unknown.

In conclusion, our study opens the path to further mechanistic investigation of the role of nociceptive signaling in the regulation of PC mobilization from the perspective of finding new therapeutic targets compatible with pain relief and cardiovascular repair.

Acknowledgments

The authors wish to acknowledge the assistance of Dr Andrew Herman and the University of Bristol Faculty of the Medical and Veterinary Sciences Flow Cytometry Facility.

Sources of Funding

This study was supported by a grant of the EU-FP7 “Resolve” and by British Heart Foundation grants for “Bone marrow dysfunction alters vascular homeostasis in diabetes.” Costanza Emanuelli is BHF senior basic research fellow and Carlotta Reni is supported by a Medical Research Council PhD studentship.

Disclosures

None.

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CLINICAL PERSPECTIVE

Pain and inflammation are generally thought of as medical problems. Treatment of these defense responses is routine in patients with myocardial and peripheral ischemia. However, blocking a defense can be harmful. It has been shown that taking nonsteroidal anti-inflammatory drugs can increase a person's risk of having a heart attack or stroke. Furthermore, morphine has been associated with higher mortality in patients with acute coronary syndrome. The present study provides novel insight into the role of the pain mediator substance P in vascular regeneration by bone marrow-derived stem cells. After ischemic injury, substance P is released from central terminals projecting to distinct brain stem centers, thus contributing to pain perception and pain-induced reactions, as well as from sensory fibers innervating the myocardium, leading to local neurogenic inflammation. In the present study, we show that substance P also contributes to mobilize stem cells from the bone marrow and to recruit them to the infarcted heart. Bone marrow cells attracted by substance P are able to promote neovascularization, thereby accelerating the healing of ischemic tissues. Conversely, genetic abrogation of substance P signaling or pharmacological inhibition of substance P release by morphine results in attenuation of both stem cell mobilization and reparative vascularization in models of ischemia. These new findings may have important clinical implications for tailoring new regenerative treatments based on stem cell recruitment by pain mediators. Nonetheless, the nociceptive signaling is also used in other biological contexts in which pain is not operant. Therefore, additional work is warranted to refine new therapeutic strategies compatible with pain relief and cardiovascular repair.

SUPPLEMENTAL MATERIAL

DETAILED AND EXPANDED METHODS

Myocardial infarction. Acute myocardial infarction (aMI) was induced in 7-8-week old male CD1 mice (Harlan) by permanent ligation of the left anterior descending coronary artery (LAD) as described.¹ Mice were anesthetized with 2,2,2 tribromo ethanol (0.3gm/kg, i.p.) and kept under artificial ventilation. The chest cavity was opened and, after careful dissection of the pericardium, LAD was permanently ligated using a 7-0 silk suture. Twenty four hours later, mice were sacrificed and peripheral blood (PB) and bone marrow (BM) from tibias and femurs were collected for assessment of neuropeptide levels, immunohistochemistry (IHC) and FACS analysis of cell antigenic profile.

Limb ischemia. With mice under tribromo-ethanol anesthesia, unilateral limb ischemia (LI) was induced using a refined procedure which consists of ligation with a 7-0 silk suture and electro-coagulation of the left femoral artery.³ Immediately after, spontaneously migrated, or SP- or CGRP-migrated GFP-BM cells were injected into three equidistant sites along the projection of the femoral artery of the ischemic adductor muscle (1×10^5 cells /30 μ L PBS/mouse). Control animals received injections of vehicle (PBS). Foot blood flow was measured immediately after and then at 3, 7, 14, 21 days after LI using a perfusion image system (Moor Instrument, laser Doppler, UK). The ratio between the blood flow measured in the ischemic and non-ischemic foot was calculated and considered as the index of blood flow recovery. At 21 days after LI, mice were anesthetized, perfused with heparinased PBS and then with 4% paraformaldehyde and ischemic muscles were removed and processed for IHC.

BM transplantation. BM cells from NK1-knockout (NK1-KO) or wild type (WT) mice were extracted from the femur and tibia. A single cell suspension was made by passing the BM through a 19-gauge needle and filtered through a 70- μ M nylon filter. After serial

washing with DMEM containing 5% fetal calf serum, cells were resuspended in the same medium at the concentration of 1×10^6 cells/200 μ L final volume.

C57BL/6 mice (n=24, Harlan, UK) were exposed to sub-lethal radiation dose (950 rads) according to the standard protocol approved by the University of Bristol and UK Home Office, under the supervision of technically qualified staff. The following day, mice were randomly assigned to receive BM cells (1×10^6 , iv) prepared as above either from NK1- KO or WT mice. Eight weeks after transplantation, PB cells of the recipients were collected from the tail vein and assessed for chimerism using β -galactosidase assay (*Calbiochem*). Briefly, after red blood cells lysis, cells were mounted on slides using cytopsin technique and stained for β -galactosidase activity following manufacture instruction. Positive cells were scored as the number of blue cells among the total cells under light microscopy. Then, mice underwent LI and were monitored for blood flow recovery for 21 days. At sacrifice, limb muscles were collected for IHC analysis of neovascularization. Cryo-sections of femurs were stained with a β -galactosidase antibody (*AbD serotec, UK*) to confirm chimerism.

Cells and cell culture

BM cell isolation. Mice were sacrificed and femurs and tibias collected in Dulbecco's Modified Eagle Medium (DMEM). Bone epiphyses were cut and BM flushed with DMEM added with penicillin (250 units), streptomycin (250 μ g) and a cocktail of protease inhibitors (Complete-Roche), using a syringe and a 25G needle. BM cells were then separated by a gentle trituration using a series of different size pipettes and needles until a cloudy and homogeneous solution was obtained. Cells were filtered through 40 μ m nylon cell strainer, pelleted by centrifugation (10 min, 300g) and used for migration experiments. In selected experiments, BM cells were depleted of mature hematopoietic cells by magnetic cell sorting using a Lineage Cell Depletion Kit (MACS, Miltenyi Biotec).

This is a magnetic labeling system for the depletion of mature hematopoietic cells, such as T cells, B cells, monocytes/macrophages, granulocytes and erythrocytes and their committed precursors from BM. Briefly, cells were magnetically labeled with a cocktail of biotinylated antibodies against a panel of “lineage” antigens (CD5, CD45R (B220), CD11b, Anti-Gr-1 (Ly-6G/C), 7-4, and Ter-119 antibodies) and Anti-Biotin MicroBeads. This labeling procedure leaves lineage negative cells untouched, thus allowing further separation/characterization of cells according to expression of PC markers, like CD117 (c-Kit) or Sca-1. Lineage negative cells were then processed for immunostaining or used in migration assays.

Primary culture of mouse dorsal root ganglia (DRG). Mouse DRG were collected from thoracic and lumbar spinal cords, minced in cold HBSS, digested in DMEM containing 1 mg/ml collagenase type 1A and 0.8 mg/mL DNase type IV for 60 min at 37°C, followed by incubation in DMEM containing 0.25% trypsin for 15 min at 37°C. After digestion, soybean trypsin inhibitor (Sigma) was added to neutralize trypsin. Neurons were pelleted, suspended in culturing medium (DMEM containing 10% fetal bovine serum, 10% horse serum, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 2 mM glutamine) dissociated by gentle trituration until the solution appeared cloudy and homogeneous, pelleted and then resuspended in culturing medium and plated on glass coverslips coated with poly-lysine (0.1 mg/mL) and laminin (5 mg/mL). After 48 hours, cells were gently washed with PBS and resuspended in fresh DMEM containing 0.25% fetal bovine serum, 0.25% horse serum, 100 U/mL penicillin, 0.1 mg/mL streptomycin, 2 mM glutamine. DRG and DRG conditioned medium (CM) were used in *in vitro* migration assays.

Human endothelial cells. Human umbilical vein endothelial cells (HUVECs) were cultured in EGM-2 (EBM-2 added with growth factors and other supplements including 2% FBS) and used in matrigel assay at P2 to P5.

Immunostaining procedures.

Bone fixation, decalcification and sectioning. Femoral bones were cleaned from muscle and connective tissue and fixed with 4% paraformaldehyde for 24 hours at 4°C. Bones were decalcified in 10% formic acid for 48 hours at 4°C, cryoprotected in 30% sucrose for 24 hours at 4°C and then processed for Optimal Cutting Temperature compound (OCT)-embedding. Histological section of marrow of 15µm thickness were cut on the longitudinal plane and mounted on poly-L-lysine-coated slides for histological analyses.

Immunohistochemistry. Histological sections of mouse femurs were incubated for 30 min at RT with PBS, 5% Normal Goat Serum and 0.3% Triton-X. Specimens were then incubated overnight at 4°C with 5% Normal Goat Serum, 0.1% triton-X and the desired concentration of primary antibodies reported in **Supplemental Table 1**. Immunoreactivity was detected by incubating the samples for 2 hours at RT, with 5% Normal Goat Serum and goat anti-rabbit Alexa-Fluor 488 secondary antibodies or by using a Fluorescein Tyramide Signal Amplification System (Perkin Elmer) according to the manufacturer's instructions. Histological sections were mounted in Prolong Gold (Invitrogen) containing DAPI and observed with a Leica SP5 AOBS confocal laser scanning microscope (Wolfson Bioimagingfacility, University of Bristol) equipped with a 40x oil objective. Images of 512 x 512 pixels were collected. For co-localization, single optical sections at the same focal plane were taken separately and the corresponding channels were merged. Antibody specificity was assessed using primary antibodies pre-absorbed with the peptides used for the immunization.

Histological sections of mouse muscle were incubated with Alexa 568-conjugated isolectin B4 to identify endothelial cells and FITC-conjugated α -vascular smooth actin (α -SMA) to identify smooth muscle cells (which are part of the arteriole walls) overnight

at 4°C. Slides were observed under a fluorescence microscope (Olympus CX41, Olympus, Southend-on-Sea, UK). Capillary density was calculated on sections stained with Isolectin B4. Arteriole density was calculated by counting and measuring the diameter of vessels stained with α -SMA in the whole section and then normalized to the muscular section total area (expressed in mm²).

Immunocytochemistry. Isolated BM cells depleted from lineage positive cells were processed for cytopspin (5min, 500g), fixed in 4% PFA, 10min at RT, blocked with Normal Goat Serum 1% and saponin 0.1%, for 30min at RT and treated with appropriate primary antibodies (see above) overnight at 4°C. Cytopspin preparations were then washed and treated with Goat Anti-Rabbit Alexa-Fluo 488 secondary antibodies for 1 hour at RT. Slides were then washed, mounted in Vectashield Mounting Medium containing DAPI and observed with confocal microscope using a 63X oil objective.

In vitro assays.

Migration assay. BM cell migration was assessed using transwell cell culture inserts with 3-5 μ m pore size filters. Freshly isolated BM cells were plated in the upper compartment (55x10³ cells/well in 24 well-plates or 2.5x10⁶ cells/well in 6 well-plates) and neuropeptides, DRG or CM added to the lower compartment of the transwell system chamber in DMEM and 0.5% FBS. Cells were allowed to migrate overnight (16 hours) in a humidified atmosphere (37°C, 5% CO₂). Cells in the upper compartment (non-migrated cells) and in the lower compartment (migrated cells) were collected and processed for immunostaining and FACS analysis. Enrichment of antigenically defined cell populations was expressed as the ratio of cells in lower and upper chambers and then normalized to control (cells treated with vehicle). In some experiments, BM cells were pretreated with NK1 and CGRP antagonists for 30 min before the experiment. In migration experiments using DRG and CM or when cells were injected in mice with LI, BM cells were obtained from transgenic mice expressing an "enhanced" green

fluorescent protein (EGFP) cDNA under the control of a chicken beta-actin promoter and cytomegalovirus enhancer (C57BL/6-Tg(CAG-EGFP)1Osb/J, Jackson Laboratory).

In vitro angiogenesis assay. In selected experiments, 3×10^4 cells/well of migrated or non-migrated cell fraction were seeded in 96-multiwell plate on top of 100 μ L jellified matrigel containing growth factors, in co-culture with 3×10^4 cells/well of HUVEC for 16 hours at 37°C. Gels were then fixed and observed. Five random view fields were photographed and the number of vessel branches counted. Counts in assays using migrated cells as a stimulus were normalized by counts obtained with respective non-migrated cells.

Western Blot analyses. Protein extracts and immunoblot analyses were performed as described.⁴ Briefly, BM lineage negative cells were starved for 2-3h in low serum medium then treated with SP, 100 nM, for 15min in RPMI medium. Cells were collected, washed and resuspended in lysis buffer (50 mM Hepes pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 25 mM NaF, 5mM NaPPi, 1% Triton X-100, 1% NP-40, 0.25% sodium deoxycholate, protease and phosphatase inhibitors) on ice. Protein extracts were separated by 10% acrylamide SDS-PAGE and then transferred to polyvinylidene difluoride membranes (PVDF, Bio-Rad Laboratories). Membranes were probed with primary antibodies against phospho-Akt (Ser473) (1:2000) and total Akt (1:5000) (Cell Signaling Technology), followed by secondary antibody horseradish peroxidase conjugated anti-rabbit (1:10,000, Sigma). Detection was carried out using either ECL or ECL Plus (Amersham).

Flow Cytometric Analyses. BM cells were treated with stem cells/progenitor cell and Lineage markers: anti-Ly6-A/E (Sca-1), anti-Cd117 (c-Kit), anti-CXCR4, anti-Flk1 (all from BD Biosciences) and anti-Lineage Mixture (mouse CD3e, CD11b, CD45R, Ly-6C/G, TER119, Caltag), diluted in PBS and BSA 1% for 15 minutes, at 4°C. Cells were washed, fixed and permeabilized, if necessary, and treated with primary antibodies (see

above), for 30min at 4°C. Cells were then washed and treated with Goat Anti-Rabbit Alexa-Fluo 488 secondary antibodies, for 30min at 4°C. BM cells were then analyzed using a FACS Canto II equipped with FACS Diva software (BD Biosciences).

Human studies. Experiments on human samples complied with the principles stated in the Declaration of Helsinki and were covered by institutional ethical approval. Patients gave written informed consent to sample collection. The procedures followed were in accordance with institutional guidelines. No sex-based or racial/ethnic-based differences were investigated as they were not relevant for the aim of the study. Expression of neuropeptide receptors on circulating PC was determined by flow cytometry analysis of PB mononuclear cells (PB-MNC) obtained through venipuncture from patients with aMI and respective age-matched healthy controls participating to an observational clinical trial on the prognostic value of circulating PC (ClinicalTrials.gov identifier: NCT01271309) (**Supplemental Tables 2**). In addition, migration assays using neuropeptide as chemoattractant, followed by flow cytometry characterization of migrated cells, were performed on PB-MNC of 6 healthy volunteers (average age, 30 years). Finally, migration assays and flow cytometry analyses were performed on MNC obtained from leftovers of fresh BM aspirate of patients participating to the cell therapy trials TransACT1&2 (Bristol Heart institute, University of Bristol), which consist of autologous transplantation of BM cells during either coronary artery bypass graft surgery or left ventricular reshaping surgery (**Supplemental Table 3**).

Human heart explants were collected from patients (n=9) who underwent cardiac transplantation 4-13 days after aMI at the University Hospital of Udine, Italy (**Supplemental Table 4**). Three of these patients had received a 2nd transplantation for an infarction of the graft, which led in 2 cases to cardiogenic shock. Samples from infarcted area, border and distant myocardium were obtained for IHC analysis of SP, NK1, CD34 and CD45 on 5µm-thick paraffin embedded sections. Tissue specimens from

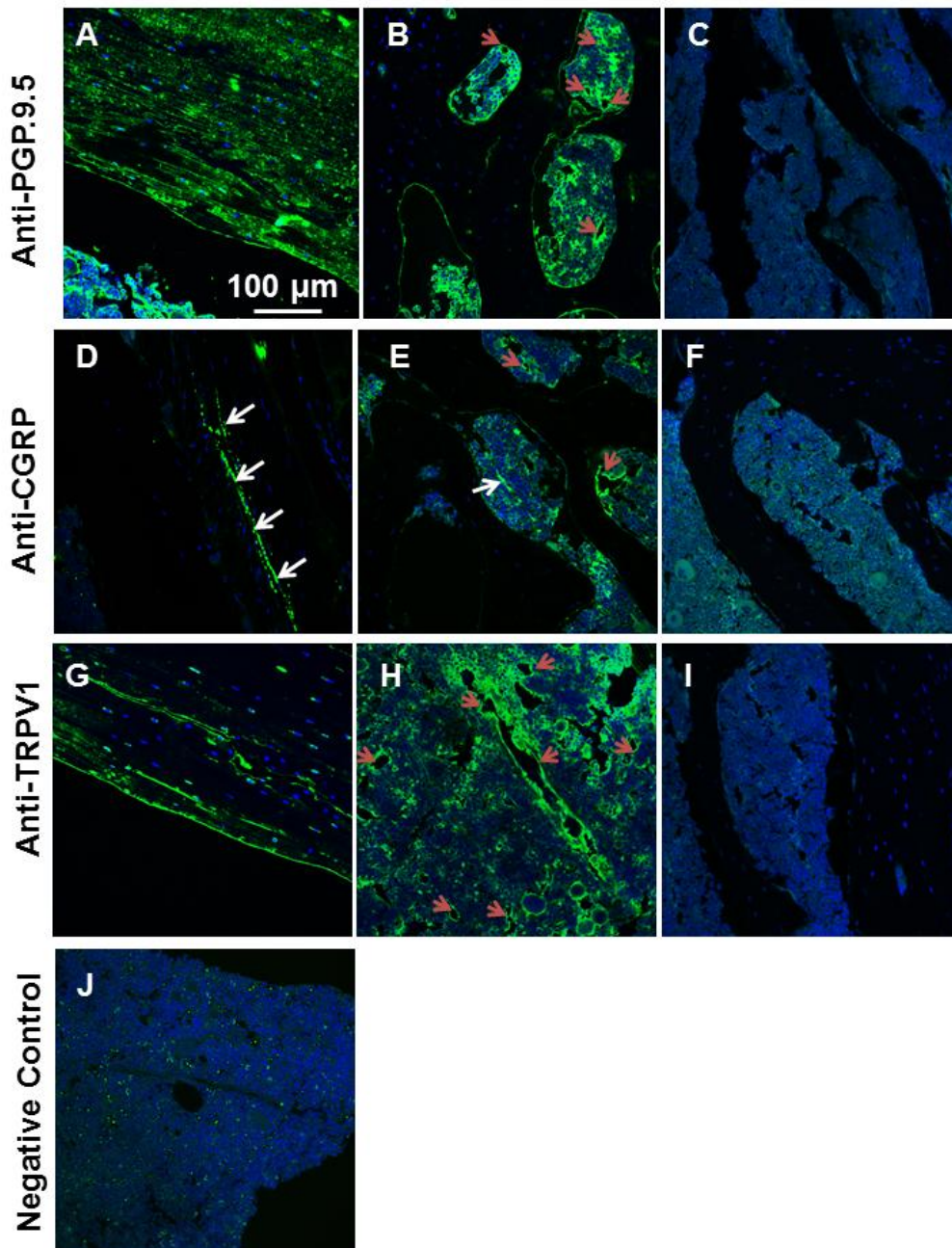
comparable areas were sampled in control normal hearts (n=5). Myocardial sampling varied from a minimum of 115 mm² to a maximum of 308 mm².

For analysis of NK1, CD34 and CD45 in the human hearts we used 5µm thick paraffin embedded sections. After deparaffinization and antigen retrieval, sections were incubated with rabbit polyclonal NK1 antibody (Santa Cruz Biotechnology, 1:100) O.N. at 4°C followed by donkey α-rabbit secondary antibody conjugated with Alexa Flour 555 (Invitrogen, Molecular Probes, 1:800) for 1 hour at 37°C. This was followed by incubation with rat monoclonal CD45 antibody (Santa Cruz Biotechnology, 1:50) for 2 hours at 37°C followed by donkey α-rat secondary antibody conjugated with DyLight 488 (Jackson ImmunoResearch, 1:200) for 1 hour at 37°C. Samples were then incubated with mouse monoclonal CD34 antibody (Dako Cytomation, 1:100) O.N. at 4°C followed by donkey α-mouse secondary antibody conjugated with DyLight 649 (Jackson ImmunoResearch) 1:400 for 1 hour at 37°C. Finally, sections were stained with DAPI to recognize the nuclei. Sections were analyzed by Leica DMI6000 B - Leica Microsystems – utilizing a 40X oil immersion objective (numerical aperture: 1.25). Final data are expressed as the number of cells per cm². For representative pictures, samples were stained with mouse monoclonal α-sarcomeric actin (Sigma-Aldrich, 1:100, 1h, 37°C). Image acquisition was carried out with a confocal laser microscope (Leica TCS-SP2, Leica Microsystems, Wetzlar, Germany), utilizing a 63X oil immersion objective (numerical aperture: 1.40) or a 40X oil immersion objective (numerical aperture: 1.25). Adobe Photoshop CS3 version 10.0 software was utilized to compose and overlay the images and adjust contrast (Adobe, USA).

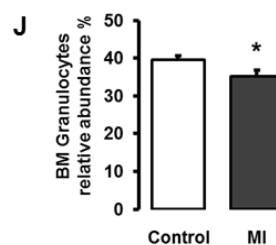
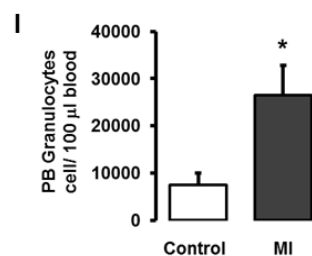
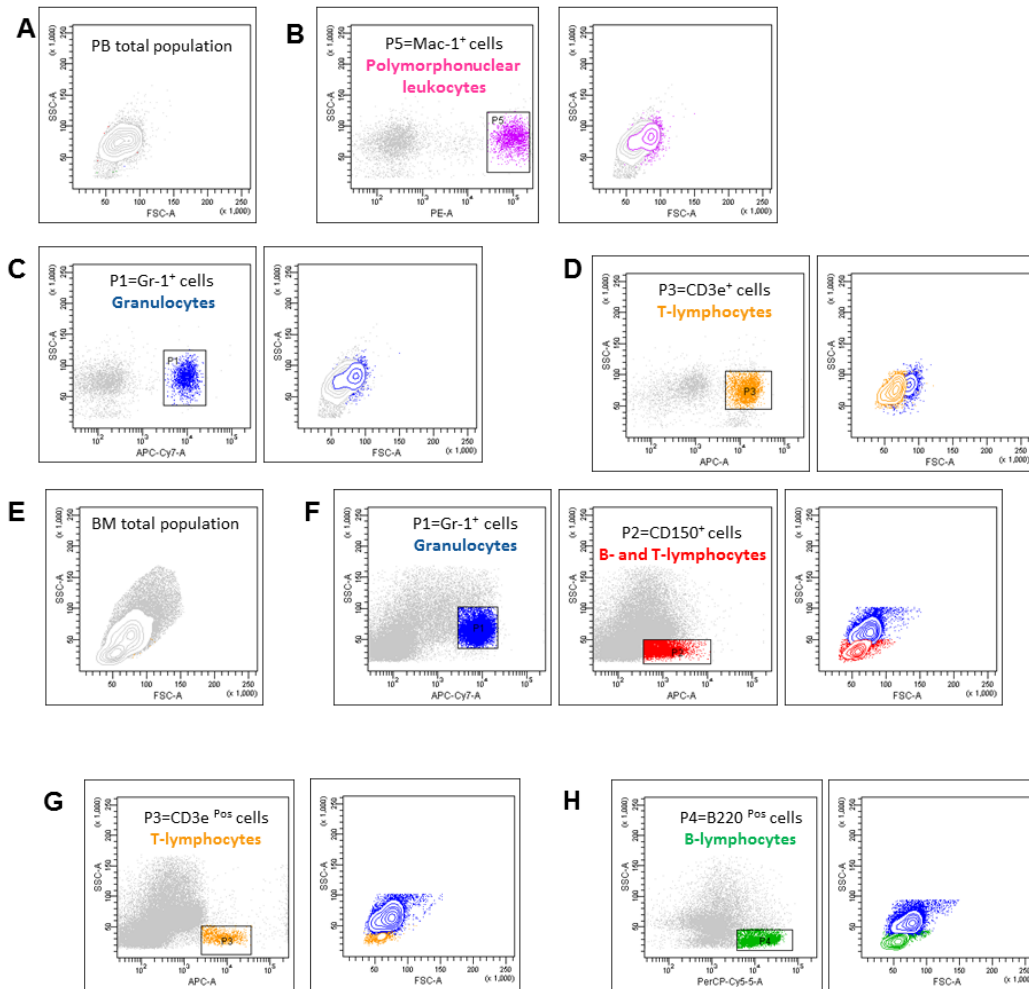
SP expression in the human hearts was identified by IHC. Specifically, 5µm thick paraffin embedded sections were deparaffinized and, after blocking endogenous peroxidase activity by 3.5% hydrogen peroxide, were incubated O/N at 4°C with a mouse monoclonal antibody (Abcam, UK, 1:2000). Detection was carried out by a

polymer-based immunohistochemical detection system (EnVision™ Detection Systems Peroxidase/DAB, Rabbit/Mouse, Dako, Denmark). Omission of the primary antibody was used as negative controls. Images were collected by Leica DMD108 digital microimaging network instrument using a 10X objective (numerical aperture 0.25) and analyzed by Image-J 1.44j software (NIH, USA). Results were expressed as fraction of myocardium expressing SP. For each sample, a total area of 10mm² was analyzed.

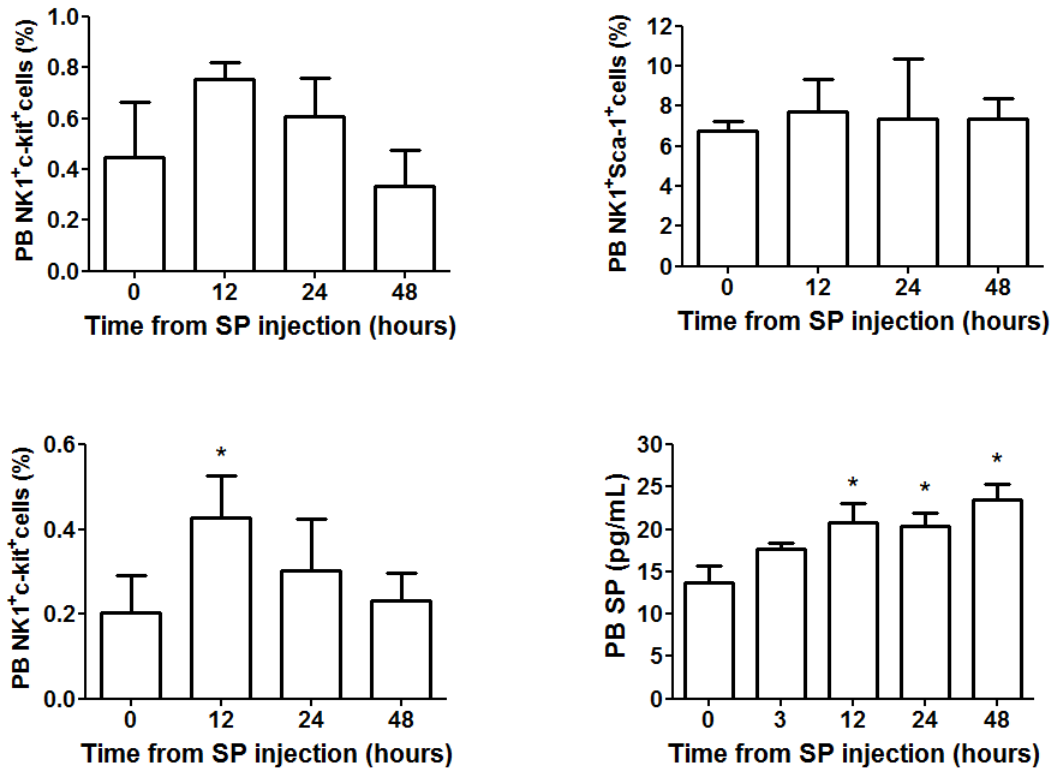
Supplemental Figure 1. Nociceptive peptidergic fibers innervate mouse bone and BM. (A) Longitudinal section of mouse femurs showing abundant innervations within bone and marrow as indicated by positive like-immunoreactivity for the pan-neuronal marker PGP9.5 at the periosteum and endosteum of the diaphysis. (B) Transversal section of the epiphysis showing PGP9.5 staining of marrow within the trabecular bone. Subsets of these fibers express CGRP (D&E) and TRPV1 (G&H). Immunostaining was not detected when the amplification step (C,F,I) or the primary antibodies were omitted (negative control, J) White arrows point to fibers, red arrows point to perivascular fibers/endothelial cells.



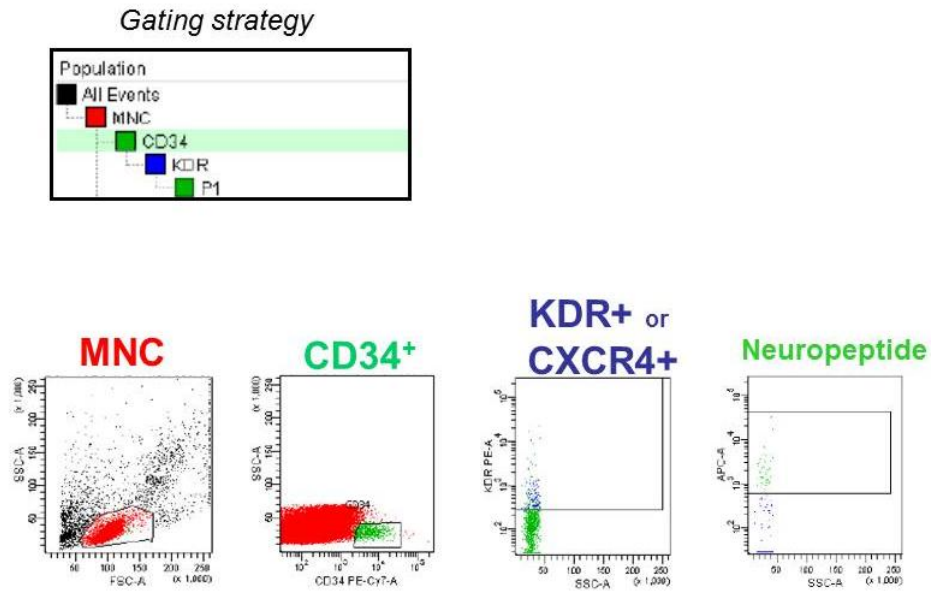
Supplemental Figure 2. Characterization of mouse BM and PB granulocytes and lympho/monocyte populations by FACS. (A-D) mouse PB cells and (E-H) mouse BM cells gate strategies and FACS analyses. Cells were fixed and permeabilized using FIX & PERM® solution according to the manufacturer. Antibodies used for characterization of BM cell fractions were the following: Mac-1 for polymorphonuclear leukocytes, Gr-1 for granulocytes; CD150 for B and T lymphocyte, CD3e for T lymphocytes and B220 for B lymphocytes. (I&J) Effect of aMI on the abundance of granulocytes in PB (I) and BM (J) (n=6, *p<0.05 vs. control).



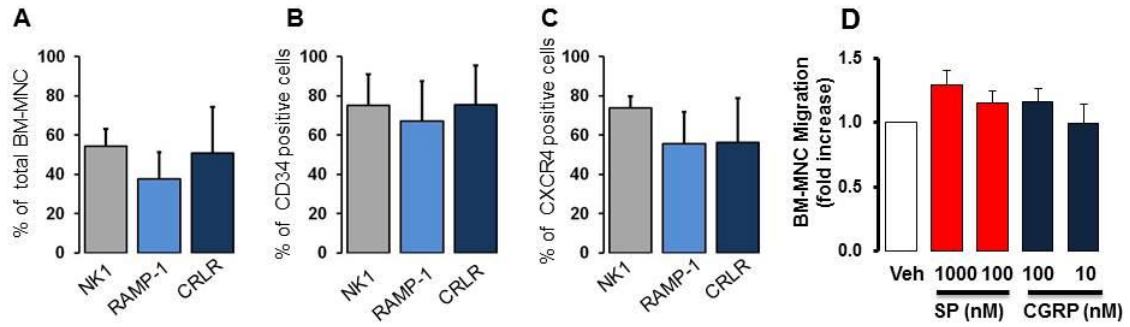
Supplemental Figure 3. Effect of SP injection on NK1-expressing PC mobilization. Bar graph showing the levels of NK1⁺c-Kit⁺ (A), NK1⁺Sca-1⁺ (B) and NK1⁺Sca-1⁺c-Kit⁺ PC (C) in PB before and after SP injection. (D) PB levels of SP before and after SP injection. *p<0.05 vs. time 0. N=3 per time point.



Supplemental Figure 4. Gating strategy for FACS analysis of human PB PC. Human mononuclear cells (MNC) were first analyzed for the expression of the progenitor cell marker CD34, then for KDR and CXCR4 and neuropeptide receptors NK1, CRLR and RAMP-1.



Supplemental Figure 5. Human BM cells express neuropeptides receptors and migrate toward neuropeptides in vitro. (A) Human BM cells express SP and CGRP receptors. **(B-C)** Human BM PC expressing CD34 and CXCR4 co-express neuropeptide receptors. **(D)** SP and CGRP induced migration of BM cells.



Supplemental Table 1. List of the antibodies

Antibody	Fluorophore	IHC Ab Conc.	ICC Ab Conc.	FACS Ab Conc.	Source
Rabbit anti-Protein gene product 9.5	Unlabelled	1:500	-	-	<i>Abcam</i>
Rabbit anti-Transient potential receptor vanilloid type 1	Unlabelled	1:250	-	-	<i>Chemicon International, MA, USA</i>
Rabbit anti-CGRP	Unlabelled	1:2500	-	-	<i>Peninsula laboratories Switzerland</i>
Rabbit anti-SP	Unlabelled	1:1500	-	-	<i>Peninsula laboratories Switzerland</i>
Rabbit anti-CRLR	Unlabelled	1:50	1:25	1:200	<i>Santa Cruz Biotechnology, CA, USA</i>
Rabbit anti-RAMP	Unlabelled	-	1:50	1:100	<i>Santa Cruz Biotechnology, CA, USA</i>
Rabbit anti-NK1	Unlabelled	1:50	1:50	1:500	<i>Immunostar, WI, USA</i>
Goat anti Rabbit	Alexa Fluor 488	1:400	1:400	1:800	<i>BD Biosciences</i>
Goat anti Rabbit	Alexa Fluor 568	-	-	1:150	<i>BD Biosciences</i>
mouse anti- CD3e, CD11b, CD45R, Ly-6C/G, TER119 (Lineage cocktail)	Alexa Fluor 488	-	-	1:100	<i>Caltag</i>
Rat anti-Ly6-A/E (Sca-1)	Phycoerythrin			1:100	<i>BD Biosciences</i>
Rat anti-CXCR4	Allophycocyanin (APC)			1:100	<i>BD Biosciences</i>
Rat anti-Flk1	APC			1:100	<i>BD Biosciences</i>
Rat anti CD45	APC			1:500	<i>BD Biosciences</i>
Rat anti-CD117 (c-Kit)	APC-eFluor780			1:400	<i>eBioscience</i>
Isolectin B4	Biotin	1:100			<i>Invitrogen</i>
α -SMA	Cy3	1:400			<i>Sigma</i>

Supplemental Table 2. Clinical data and pharmacological treatment of aMI patients and controls

	Controls (n=10)	aMI (n=10)
Patient Characteristics		
Age	69±4	64±2
Male gender	6	6
Smoker	3	3
Hypertension	4	3
Diabetes	2	0
Days from aMI	NA	1 to 6
Therapy for aMI		
Nitrates (iv)	NA	1
Heparin (iv)	NA	4
Aspirin	NA	3
Clopidogrel	NA	3
Glycoprotein IIb/IIIa receptor inhibitors	NA	1

Supplemental Table 3. Clinical data of 4 patients of the TransAct 1&2 trials that provided BM samples for FACS analyses.

Patient Characteristics	
Age	62±2
Male gender	2
Smoker	1
Hypertension	1
Diabetes	0
Previous myocardial infarct	4

Supplemental Table 4. Clinical data of patients studied for expression of SP and NK1 in the heart.

Patient Characteristics	Patients transplanted following an AMI	Patients re-transplanted following an aMI of the graft	Controls
n	6	3	5
Male gender	6	2	2
Age (years)	56±6	54±14	44±18
Time from aMI to transplant (days)	9±6	4±1	
Body Weight (Kg)	76.8±8.0	66.7±2.1	
Height (cm)	173±5	173±10	
BMI	25±1	23±2	
Smoke (n)	4	1	
Dyslipidemia (n)	4	N/A	
Hypertension (n)	4	N/A	
NYHA class (n)	IV (6)	IV (2) III (1)	

Continuous data are presented as mean±SD. N/A: not assessed

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