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Nitric Oxide Is a Regulator of Hematopoietic Stem Cell Activity

Tatyana Michurina,[1] Peter Krasnov,[1] Alejandro Balazs,[2] Naoki Nakaya,[1] Tamara Vasilieva,[3] Boris Kuzin,[1,3] Nikolay Khrushchov,[3] Richard C. Mulligan,[2] and Grigori Enikolopov[1,*]

[1] *Cold Spring Harbor Laboratory, Cold Spring Harbor, NY 11724, USA*
[2] *Department of Genetics, Children's Hospital, Harvard Medical School, Boston, MA 02115, USA*
[3] *Institute of Developmental Biology, Russian Academy of Sciences, Moscow, Russia*

**To whom correspondence and reprint requests should be addressed at Cold Spring Harbor Laboratory, 1 Bungtown Road, Cold Spring Harbor, NY 11724, USA. Fax: (516) 367-6805. E-mail: enik@cshl.edu.*

Hematopoietic stem cells give rise to various multipotent progenitor populations, which expand in response to cytokines and which ultimately generate all of the elements of the blood. Here we show that it is possible to increase the number of stem and progenitor cells in the bone marrow (BM) by suppressing the activity of NO synthases (NOS). Exposure of mice to NOS inhibitors, either directly or after irradiation and BM transplantation, increases the number of stem cells in the BM. In the transplantation model, this increase is followed by a transient increase in the number of neutrophils in the peripheral blood. Thus, our results indicate that NO is important for the control of hematopoietic stem cells in the BM. They further suggest that suppression of NO synthase activity may allow expansion of the number of hematopoietic stem and progenitor cells or neutrophils for therapeutic purposes.

Key Words: hematopoiesis, stem cells, nitric oxide, differentiation, bone marrow, transplantation

INTRODUCTION

Cells produced by the hematopoietic system of the adult organism originate from rare pluripotent hematopoietic stem cells that reside in the bone marrow (BM) [1,2]. In contrast to the rapid division of progenitor cells, the proliferative activity of hematopoietic stem cells in the BM is restricted [3–8]. The relative quiescence of the pool of stem cells and its resistance to cytokine signaling may be crucial for preventing the premature depletion of the stem cell compartment during stressful conditions. The signaling systems that control the pool of hematopoietic stem cells are poorly understood, and so far only a handful of regulators of hematopoietic stem cells has been described [7–12].

NO, a multifunctional signaling molecule [13], can induce gene expression, affect multiple signaling pathways, and act as a potent antiproliferative factor in a variety of cells; thus, it may potentially contribute to the homeostasis of the stem cell pool. Expression of various NO synthase (NOS) isoforms has been reported in human and rodent BM and blood [14]: for instance, iNOS RNA can be detected in megakaryocytes, eosinophils, and unstimulated monocytes [15,16]; eNOS RNA has been found in platelets, megakaryocytes, and lymphocytes [17,18]; and nNOS RNA has been detected in neutrophils [15,18,19]. Furthermore, inhibitory action of exogenous NO donors on *in vitro* colony growth of various subpopulations of BM cells has been demonstrated for human BM cells; for instance, NO donors can markedly decrease production of myeloid and erythroid colonies by $CD34^+$ cells [20,21]. Moreover, NO may mediate the action of some cytokines (e.g., TNFα and IFNγ) *in vitro* [20,22]. However, its contribution to the control of hematopoietic stem or progenitor cells during normal hematopoiesis in the organism has not yet been explored. We show here that inhibition of NOS activity *in vivo* results in an increase in the number of stem and early progenitor cells in the BM. Moreover, we demonstrate that this increase in the number of stem cells is followed by an increase in

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the neutrophil content in the blood. Our results point to NOS as an important regulator of hematopoietic stem cells and indicate that it may be possible to expand the number of cells in the stem cell pool by suppressing the activity of NOS.

RESULTS

To investigate the potential contribution of NO to hematopoiesis, we first determined if NO synthases are expressed in cells of the mouse BM. We found that mRNA of each NOS isoform (neuronal, endothelial, and inducible, or nNOS, eNOS, and iNOS) can be reliably detected in the BM using conventional and quantitative real-time RT-PCR (Fig. 1a and data not shown); however, the levels of the corresponding proteins are below the threshold for detection by Western blot analysis (data not shown).

To examine the possible role of NO in regulating hematopoiesis, we treated animals with specific inhibitors of NOS activity and applied a series of assays to probe various stages of hematopoiesis. We used two structurally unrelated compounds, N^{ω}-nitro-L-arginine methyl ester (L-NAME) and 2-ethyl-2-thiopseudourea hydrobromide (ETU), which are potent inhibitors of mammalian NO synthases; for controls, we used saline or N^{ω}-nitro-D-arginine methyl ester (D-NAME), an inactive enantiomer of L-NAME.

To examine whether NOS inhibitors affect the survival of cells in the BM, we monitored the fraction of dead cells by staining with trypan blue and the fraction of apoptotic

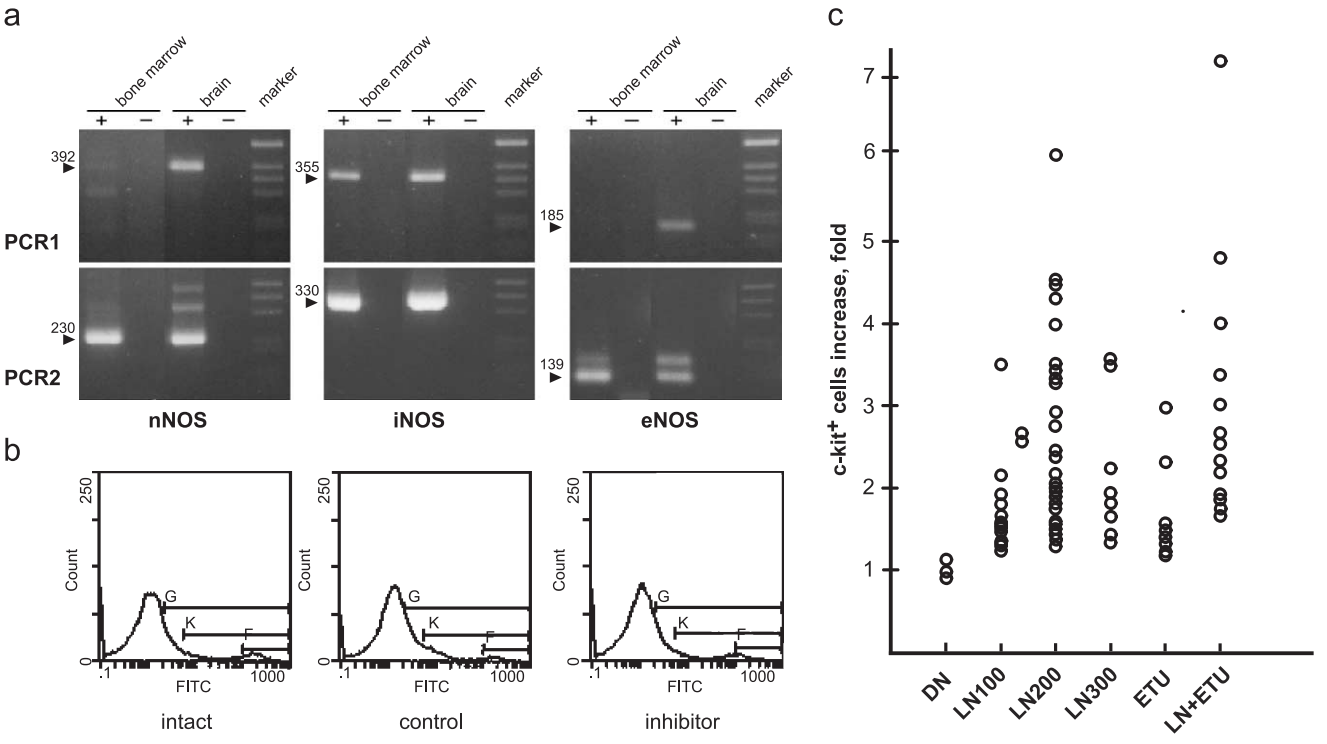

FIG. 1. (a) Three NOS isoforms are expressed in the BM. Total RNA from the mouse BM and brain was used to synthesize cDNA using an oligo(dT) primer; cDNA was amplified by PCR using NOS isoform-specific primers (PCR1). An aliquot of the product from the first round was used for a second round of amplification with another set of primers (seminested PCR; PCR2). In control experiments reverse transcriptase (RT) was omitted (–). The resulting RT-PCR products (sizes indicated by arrowheads) are reliable representations of each NOS gene transcript tested, since they were reproducibly obtained with several combinations of primers for each gene, produced DNA fragments of the expected nucleotide sequence, and generated DNA fragments of the expected size after digestion with restriction endonucleases (not shown). For the nNOS and eNOS mRNA in the BM, the RT-PCR products were detectable only after the second round of amplification, indicating that their transcripts are rare in the RNA preparation from total BM. (b) Treatment with NOS inhibitors does not induce toxicity or apoptotic death in BM cells. For the analysis of apoptosis, total mononuclear BM cells from irradiated recipients, which received NOS inhibitors or control solutions for 16 days after BM transplantation, and from intact animals were fixed and permeabilized with ethanol. Apoptotic cells were labeled by TUNEL reaction with a fluorescein label and analyzed by FACS. For negative control, the terminal deoxynucleotidyl transferase was omitted; for positive control, the cells were treated with DNase I before labeling. The histograms shown are representative examples of at least two independent experiments. (c) Treatment with NOS inhibitors increases the proportion of progenitor cells in the BM. Changes in the content of c-kit^{+} cells as determined by FACS in the bone marrow of mice treated with NOS inhibitors after transplantation as indicated: DN, 200 μg/g D-NAME; LN100, 100 μg/g L-NAME; LN200, 200 μg/g L-NAME; LN300, 300 μg/g L-NAME; ETU, 20 μg/g ETU; LN + ETU, 300 μg/g L-NAME and 30 μg/g ETU.

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cells by the TUNEL assay. Neither L-NAME, nor ETU, nor their combination at the concentrations used in these experiments induced noticeable apoptotic death (Fig. 1b) or toxicity: the fraction of nonviable cells as determined by exclusion of trypan blue was similar in mice treated with saline or NOS inhibitors L-NAME and ETU for 4 h (10.1% vs 9.9% of the total BM cell preparation) or 24 h (6.2% vs 4.7%) after injection and in mice injected with NOS inhibitors for 12 days (10.6% vs 9.8%). Furthermore, prolonged exposure to NOS inhibitors did not noticeably affect the viability of the animals (data not shown).

To estimate the effective concentration of the NOS inhibitors, we exposed animals to various concentrations of NOS inhibitors after BM transplantation and determined expression of the cell surface marker c-kit, which is expressed by several classes of hematopoietic progenitors in the BM. The proportion of c-kit$^+$ cells in the BM was increased after treatment with either of two NOS inhibitors (L-NAME and ETU); it was further augmented by increasing the concentration of L-NAME or by combining L-NAME with ETU, and it was not observed when D-NAME or saline was used (Fig. 1c). These observations support the notion that specific inhibition of NOS was the cause of the observed changes. The increase in c-kit$^+$ cells continued for 2–3 days after the treatment with NOS inhibitors was suspended; this difference disappeared 7–8 days after the treatment was ended (data not shown).

We used two experimental settings to study the possible role of NO in hematopoiesis. In the first, we injected mice with NOS inhibitors or control solutions and then analyzed the changes in BM and peripheral blood (PB). In the second, we used a model of BM transplantation into syngeneic lethally irradiated recipient mice that were treated, after transplantation, with inhibitors of NOS or control solutions.

To examine the effects of suppression of NOS activity on hematopoietic stem cells, we treated animals with NOS inhibitors or with control saline solution for 6 or 12 days and analyzed the changes in long-term engraftment of cells from these animals using a competitive repopulation assay: we mixed BM cells from the treated animals expressing the Ly-5.1 (CD45.1) alloantigen with a 4-fold excess of competitor cells from mice of a congenic line that expresses Ly-5.2 (CD45.2) alloantigen and transplanted the mixture into lethally irradiated Ly-5.2 recipient mice. We analyzed the PB of the recipients for the content of Ly-5.1 and Ly-5.2 cells 2, 3, and 6 months after transplantation. When mice were treated with NOS inhibitors for 6 days, their BM cells were more efficient in their repopulating ability than cells from the control animals (2.4-fold difference when tested after 6 months). The difference was not evident after 12 days of exposure to NOS inhibitors (Fig. 2a) (perhaps indicating that at this time point the effect of the inhibitors is suppressed or compensated).

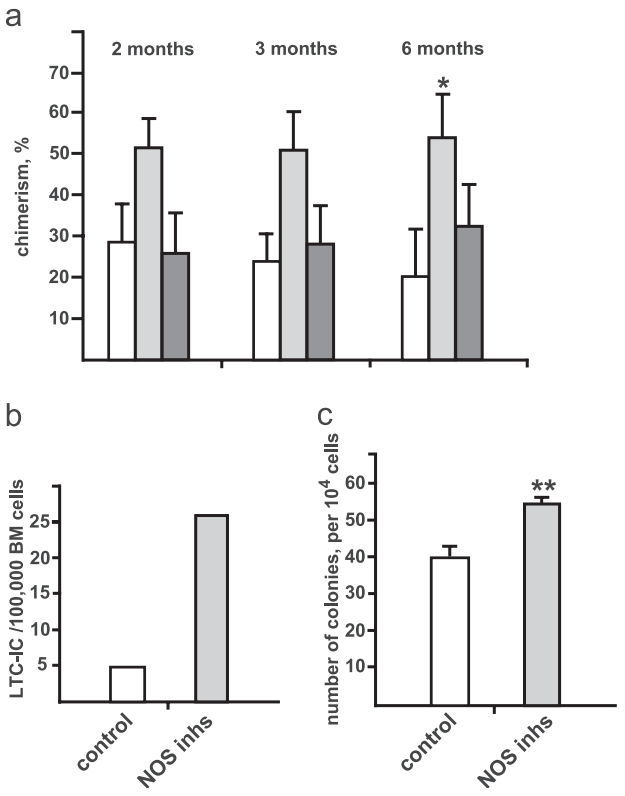

FIG. 2. Inhibition of NOS increases the number of stem and progenitor cells in the BM. (a) Competitive repopulation assay. Ly-5.1 (CD45.1) animals were treated with NOS inhibitors or control solutions for 6 or 12 days. 50×10^3 BM cells were mixed with 200×10^3 BM cells from Ly-5.2 (CD45.2) animals and transplanted into the irradiated secondary recipients of the Ly-5.2 genotype. 2, 3, and 6 months after transplantation PB was collected from the retro-orbital venous plexus and analyzed by FACS for the relative contribution of donor Ly-5.1 cells. Open bars represent data for the control group, shaded bars—animals treated with NOS inhibitors for 6 days, dark shaded bars—animals treated for 12 days. (b) LTC-IC assay. Animals were treated with NOS inhibitors or control solutions for 9 days. The number of LTC-IC cells in the BM was assessed using limiting dilution analysis after growing the cells on the stromal cell layer and replating in methylcellulose for colony formation. Data are presented as the number of LTC-IC per 1×10^5 BM cells taken for analysis. (c) CFC assay. BM cells from animals treated with NOS inhibitors or control solutions for 9 days were isolated 3 days later and were cultivated for 12 days in methylcellulose. For (b) and (c), open bars represent data for the control group, shaded bars—animals treated with NOS inhibitors. Data were analyzed using *t* test; $*P < 0.05$, $**P < 0.01$.

The increase in the number of stem cells after treatment with NOS inhibitors observed in the *in vivo* test was also evident in the *in vitro* LTC-IC assay. In this test we used limiting-dilution analysis to estimate the frequency of LTC-ICs, which correspond to primitive stem cells in the BM. There were 5.4-fold more LTC-IC units in the BM of mice exposed to NOS inhibitors for 9 days than in control mice (1 in 3891 vs 1 in 21,167) (Fig. 2b). Furthermore, the colony-forming ability of the LTC-IC cells in the bulk culture assay was also significantly increased after treatment with NOS inhibitors (1.75-fold increase in

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the total number of colonies and 4.5-fold increase in macroscopic colonies, $P < 0.05$). We next examined the changes in the number of committed progenitors by comparing the total number of colony-forming cells (CFC) in the BM of control animals and animals exposed to NOS inhibitors. There was a 1.4-fold increase in CFCs capable of initiating colony growth in methylcellulose in animals treated with inhibitors than in the controls (Fig. 2c); importantly, this increase was transient and disappeared 8 days after the cessation of treatment. Finally, we examined if exposure to NOS inhibitors results in changes in the PB. We were unable to detect changes in the parameters we analyzed (hematocrit, content of erythrocytes, total leukocytes, differential leukocyte count) in response to NOS inhibitors 0, 7, or 42 days after cessation of treatment.

We then analyzed the effects of NOS inhibitors under conditions under which the endogenous pool of hematopoietic cells is ablated by irradiation. This allows us to follow a limited number of transplanted stem cells under conditions that demand an enhanced activity of stem cells. Thus, in this setting, we irradiated recipient mice (primary recipients), transferred BM cells from donor animals, and then treated the recipients with NOS inhibitors.

We again used the competitive repopulation assay to examine long-term engraftment in this setting. Thus, in these experiments we transplanted cells from Ly-5.1 donors to Ly-5.1 primary recipients, treated the recipients with NOS inhibitors, mixed their BM cells with the competitor cells from Ly-5.2 mice, and transplanted the cells into Ly-5.2 animals (thus, in this setting, secondary recipients); 3, 5, and 7 months later we analyzed blood from the secondary recipients for the Ly-5.1/Ly-5.2 ratio. BM cells from animals that received NOS inhibitors for 9 days after BM transplantation (i.e., the primary recipients) were 8–12 times more efficient in restoring the hematopoietic system of the secondary recipients than cells from control animals (Fig. 3a). This

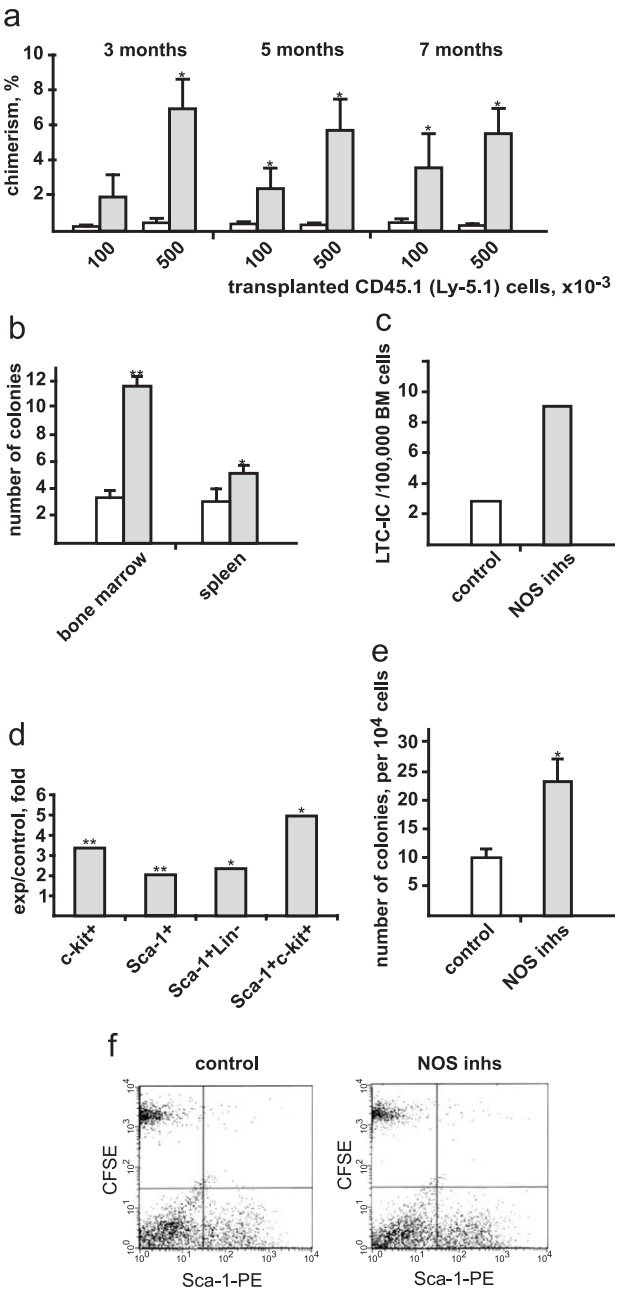

FIG. 3. Inhibition of NOS after irradiation and transplantation increases the number of stem and progenitor cells in the BM. (a) Competitive repopulation assay. Ly-5.1 (CD45.1) animals received irradiation and BM transplantation and were treated with NOS inhibitors or control solutions for 9 days. 100×10^3 or 500×10^3 BM cells were mixed with 200×10^3 BM cells from Ly-5.2 (CD45.2) animals and retransplanted into the irradiated secondary recipients of the Ly-5.2 genotype. 3, 5, and 7 months after transplantation PB was collected from the retro-orbital venous plexus and analyzed by FACS for the relative contribution of donor Ly-5.1 cells. (b) Pre-CFU-S assay. Animals were treated with NOS inhibitors L-NAME and ETU or control solution after BM transplantation. After 12 days of treatment BM cells and spleen cells were isolated and retransplanted into the irradiated secondary recipients. 12 days after retransplantation the number of colonies in the spleens of the secondary recipients (per 1×10^5 for the transplanted BM cells and per 1×10^6 for the transplanted spleen cells) was counted. (c) LTC-IC assay. Animals were treated with NOS inhibitors or control solutions for 9 days. The number of LTC-IC cells in the BM was assessed using limiting-dilution analysis after cells were grown on the stromal cell layer and replated in methylcellulose for colony formation. Data are presented as the number of LTC-IC per 1×10^5 BM cells taken for analysis. (d) Flow cytometry analysis of the distribution of surface markers (Sca-1, c-kit, and lineage markers) in the BM of control mice (treated with saline for 7–10 days after BM transplantation) and experimental animals (treated with NOS inhibitors for 7–10 days). Data are presented as the ratio of the percentage of the indicated class of cells in experimental animals to that in control animals. (e) CFC assay. BM cells from animals treated with NOS inhibitors or control solutions for 9 days were cultivated for 12 days in methylcellulose. (f) Homing assay. Donor cells were stained with CFSE and injected into irradiated mice. Recipient mice were treated with NOS inhibitors or saline. BM was isolated 20 h after injection and analyzed by flow cytometry for $CFSE^+$ $Sca\text{-}1^+$ Lin^- cells. Note that dot plots represent Lin^- cells. For all graphs, open bars represent data for the control groups, shaded bars—animals treated with NOS inhibitors. Data were analyzed using *t* test; $^*P < 0.05$, $^{**}P < 0.01$.

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increase was seen at different ratios of Ly-5.1 to Ly-5.2 cells in the transplanted mixture and at different times after transplantation.

The changes induced by NOS inhibitors were also evident when we used another retransplantation-based *in vivo* test, the pre-CFU-S assay. After irradiation, BM transplantation, and treatment of the recipients with NOS inhibitors or control solutions, we transferred the BM or spleen cells of these mice into irradiated secondary recipients and determined the number of day 12 spleen colonies. The number of pre-CFUs in the BM of animals treated with inhibitors (as measured by the frequency of CFU-S in the secondary recipients) was 3.5-fold higher than in controls (Fig. 3b). A smaller, but significant, increase over the controls was also seen when spleen cells from treated animals were transplanted into secondary recipients.

The increase in the number of stem cells after transplantation and treatment with NOS inhibitors was also evident in the LTC-IC assay: there were 3.1-fold more LTC-ICs in the BM of mice exposed to inhibitors for 9 days than in control mice (1 in 11,129 vs 1 in 34,680) (Fig. 3c). As in the first setting, the colony-forming ability of the LTC-IC cells in the bulk culture assay was also increased by NOS inhibitors (2.6-fold increase in the total number of colonies and 4.9-fold increase in macroscopic colonies, $P < 0.05$). Furthermore, the changes induced by inhibitors after transplantation were also apparent when the distribution of surface markers expressed by hematopoietic stem and progenitor cells was examined: fractions of c-kit$^+$, Sca-1$^+$, Sca-1$^+$Lin$^-$, and Sca-1$^+$c-kit$^+$ cells were elevated 3.4-, 2.1-, 2.4-, and 5.0-fold, respectively (Fig. 3d). Finally, the fraction of committed progenitors revealed in the CFC assay was 2.3-fold higher in the BM of animals that were treated with NOS inhibitors after transplantation, compared to the control (Fig. 3e).

To examine whether the observed effects may reflect altered homing of transplanted cells in the presence of NOS inhibitors, we measured the distribution of BM cells labeled with 5-(and 6)-carboxyfluorescein diacetate, succinimidyl diester (CFSE) and transplanted into the irradiated animals, which then received injection of NOS inhibitors or saline. Exposure to NOS inhibitors did not change the distribution of CFSE$^+$Sca-1$^+$Lin$^-$ cells in the BM, indicating that homing to the BM was not affected by the treatment (Fig. 3f).

Together, these data demonstrate that inhibition of NOS activity after transplantation results in an increase in the proportion of stem and early progenitor cells in the BM. We then asked whether the changes in the BM revealed in this experimental setting are followed by changes in the PB of the treated animals. We treated animals with NOS inhibitors after transplantation and then monitored the composition of the PB at various times after the cessation of the treatment. The number of erythrocytes and platelets did not vary between treated

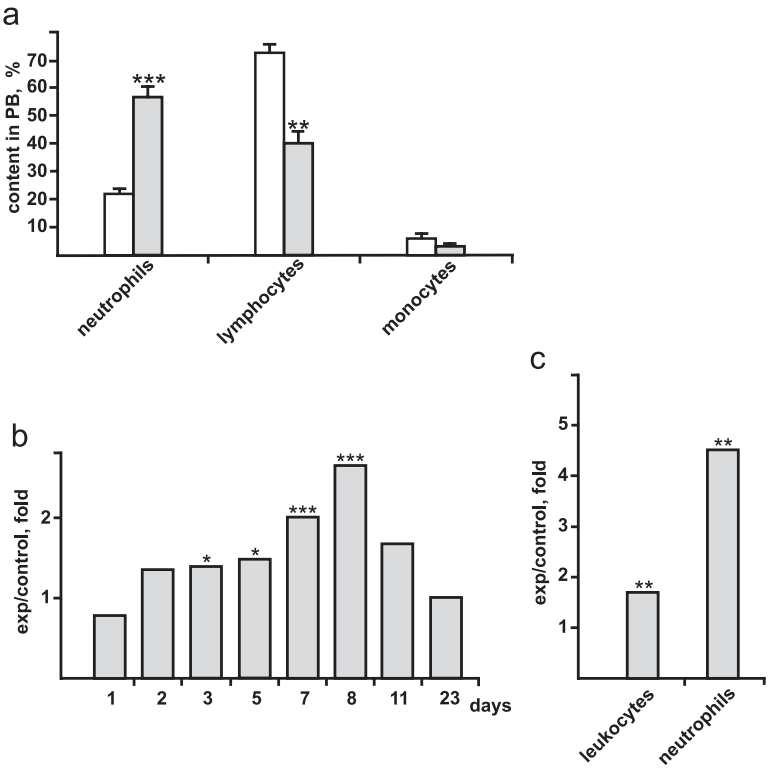

FIG. 4. Inhibition of NOS activity increases the neutrophil content in the blood. Animals were treated with control solution or with NOS inhibitors for 9 days. Blood was taken 8 days (a and c) or at the indicated times (b) after the cessation of treatment and a differential leukocyte count was performed. Data were analyzed using *t* test; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. (a) Differential leukocyte count. Open bars represent data for the control group, shaded bars—animals treated with NOS inhibitors. (b) Changes in the number of neutrophils. Blood was taken at the indicated times after the cessation of treatment (1 to 23 days, *x* axis) and a differential leukocyte count was performed to determine the percentage of neutrophils. The ratio of the percentages in the experimental vs control animals are plotted on the *y* axis. (c) Changes in the total numbers of leukocytes and neutrophils. The total leukocyte count and the content of neutrophils in experimental and control animals were determined and are presented as ratios. Note that in (c) the total neutrophil content is analyzed, whereas in (a) and (b) the fraction of neutrophils among leukocytes is analyzed.

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and control groups at 8, 39, or 90 days after treatment with inhibitors. However, the number of leukocytes was 1.7-fold higher in animals treated with NOS inhibitors compared to the controls 8 days after the cessation of treatment; the difference then diminished and the leukocyte count was equal in experimental and control groups 39 and 90 days after the cessation (Fig. 4c and data not shown). Differential leukocyte count of the PB showed that 8 days after the treatment, the fraction of neutrophils was 2.6 times higher in treated compared to control animals (Fig. 4a), whereas the fractions of lymphocytes and monocytes decreased (0.56 and 0.52 of the control levels, respectively). The increase in neutrophils was gradual and then diminished, so that 23 days after the exposure to inhibitors, the fraction of neutrophils was the same in the control and experimental groups (Fig. 4b). This increase in the fraction of neutrophils was augmented by the increase in the total number of leukocytes, such that 8 days after the cessation of treatment the content of neutrophils in the blood of the treated animals was 4.5-fold higher than in control animals (Fig. 4c). Thus, the increase in the content of stem and progenitor cells in the BM that is induced by NOS inhibitors is closely followed by a transient increase in the neutrophil content in the blood.

Discussion

Our experiments demonstrate a novel role for NO in controlling stem and early progenitor cells in the BM. We show that when NOS activity is suppressed, there is an increase in the number of stem and progenitor cells in the BM. This effect is particularly pronounced in the model of BM transplantation, possibly reflecting an increased demand for hematopoiesis after ablation of the endogenous hematopoietic cell pool. In this context, the changes in stem cells in the BM are followed by an increase in the number of neutrophils in the blood. Together, our results indicate that NO is an important regulator of hematopoiesis.

Our results are compatible with the notion that NO acts to control proliferation of hematopoietic stem cells in the BM. There are numerous observations, both *in vitro* and *in vivo*, that support a role for NO in negative regulation of stem cell division. NO has been shown to mediate the action of growth factors and to control the balance between proliferation and differentiation in cultured cells, including neuronal and endothelial cells, cardiomyocytes, adipocytes, and osteoblasts [23]. It has been also demonstrated that exposure to chemical donors of NO can change the extent of hematopoietic maturation *in vitro* [20,21] and that NO mediates the action of TNFα, IFNγ, and GM-CSF on hematopoietic cells *in vitro* [20–22,24]. *In vivo*, NO has been shown to act as an essential antiproliferative signal during *Drosophila* disc development [25–27], moth neurogenesis [28], and *Xenopus* brain development [29], as well as in the adult mammalian brain [30–32]. Thus, in a variety of contexts, NO may be acting as a reversible ''damper'' to slow down or arrest the cell cycle. This activity of NO may overlap with another recently described role for NO in another hematopoiesis-related process, the mobilization of stem and progenitor cells to the PB [33].

Our results may indicate that NO helps to establish a dominant antiproliferative tone [8] in stem cells, thus contributing to their quiescence. NO could help restrain stem cells from cycling since NOS expression is induced by many types of cytokines that affect BM. In this model, NO may be produced either by stem cells, in an autocrine fashion, or by neighboring cells, e.g., stromal or differentiated hematopoietic cells, in a paracrine fashion. The inhibitory tone maintained with the help of NO may protect the stem cell pool from premature exhaustion and depletion under conditions of stress.

Potential effectors of NO in the BM may include the cyclin-dependent kinase inhibitor p21WAF1: in the absence of p21, proliferation of hematopoietic stem cells is increased [7,8], and NO is known to induce p21WAF1 expression in several systems [34–38]. Other effectors of NO signaling might include HOXB4 or Bmi-1, which were recently described as critical regulators of stem cells pools in animals [9–12]. Yet another possible link between NO and hematopoiesis may emerge from the potential of NO to regulate proteolysis and, thus, to affect the mobilization of stem cells or their interaction with the stromal cells in the BM [33,39]. The mechanistic link between NO and hematopoiesis may involve, more generally, modification of the activity of proteins [40,41], redox regulation [41,42], and resetting gene expression patterns in the cell by activating multiple signaling pathways [38]; thus, suppression of NOS activity may induce profound changes in hematopoietic stem cells and increase their self-renewal or survival potential.

An important observation from our experiments is that, after BM transplantation, the NOS inhibitor-induced increase in the number of stem and progenitor cells in the BM is followed by an increase in the number of neutrophils in the blood. This implies that after NOS inhibition is suspended, the extra progenitor cells of the BM successfully proceed through hematopoietic differentiation and appear in the PB as mature neutrophils. Our results point to NOS as an important pharmacological target in the BM and suggest that inhibition of NOS activity may find therapeutic utility for *in vivo* or *ex vivo* expansion of hematopoietic stem and progenitor cell populations.

Materials and Methods

Animals. Six- to 12-week-old C57Bl/6 and C57Bl/B6SjlPep3 female and male mice (Jackson Laboratories, Bar Harbor, ME, USA, or Taconic Farms, Germantown, NY, USA) were used. All mice were bred and maintained at

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the CSHL Animal Care Facility in microisolator cages and provided with autoclaved standard food diet and acidified water ad libitum.

Antibodies. PE-conjugated monoclonal antibodies (mAb) against Sca-1 (E13-161.7) and CD45.1 (Ly-5.1; A20); FITC-conjugated mAb against c-kit (2B8), CD45.2 (Ly-5.2; 104), and lineage markers CD11b (M1/70), CD5 (53-7.3), CD45R (RA3-6B2), and Gr-1 (RB6-8C5); mAb against CD16/CD32 (Fc block, 2.4G2); and streptavidin-PerCP were from BD Pharmingen (San Diego, CA, USA). StemSep murine hematopoietic enrichment cocktail containing mAb against lineage markers CD5, CDE45R, CD11b, Ter119, Gr-1, and 7-4 was from StemCell Technologies (Vancouver, BC, Canada).

RT-PCR. Total RNA was extracted from $4-40 \times 10^6$ total BMC, Sca-1$^+$ cells, or brain, using TRIzol reagent (Gibco BRL, Gaithersburg, MD, USA) according to the manufacturer's instructions. cDNA was synthesized from 1.5 μg of total RNA using an oligo(dT) primer and MuLV reverse transcriptase (Applied Biosystems, Foster City, CA, USA). For negative controls, reverse transcriptase was omitted. One-half of each RT reaction was used for the first round of amplification (PCR1). For the second round (seminested PCR; PCR2), 1/10 of the PCR1 reactions was used. For all three NOS isoforms, the amplifications were performed as follows: for PCR1, 45 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 1 min; for PCR2, 30 cycles of 95°C for 30 s, 56°C for 30 s, and 72°C for 1 min. For nNOS, PCR1 was performed with forward primer 5′-TTTGGCTCCAAGGCCCACATG-3′ from exon 6 and reverse primer 5′-TGTGAACTCCACATTAGCTGG-3′ from exon 8; in PCR2, the same reverse primer was used, but forward primer was from exon 7, 5′-GTGTTCGATGCCCGAGACTGC-3′. For iNOS, PCR1 was performed with forward primer 5′-GATGTCCGAAGCAAACATCACATTCAGA-3′ from exon 18 and reverse primer 5′-AGGCACACAGTTTGGTGT-GGTGTA-3′ from exon 20; in PCR2, the latter was replaced with reverse primer 5′-GACAATCCACAACTCGCTCCAA-3′ from the same exon. For eNOS, PCR1 was performed using forward primer 5′-GCCTTCCAGGCTGCCTGTGA-3′ from exon 17 and reverse primer 5′-GATTGTAGCCTGGAACATCTTCCG-3′ from exon 18; in PCR2, this reverse primer was replaced with reverse primer 5′-GGTAGTAATTG-CAGGCTCTCAG-3′ from exon 17. Exon borders were mapped by homology search of mouse NOS mRNAs with corresponding exon sequences of human (nNOS and eNOS) and rat (iNOS) genes.

BM cell isolation. Mice were sacrificed by cervical dislocation and BM cells were isolated from femurs and tibiae by flushing with minimal essential medium (Gibco BRL) supplemented with 2% heat-inactivated fetal bovine serum (FBS; Gibco BRL).

Irradiation and BM transplantation. Recipient mice were exposed to 9.0–11.0 Gy total body γ irradiation using the Marc I irradiator with cesium-137 source (Atomic Energy of Canada, Ottawa) at a dose rate of 1.06 Gy/min 20 h before BM transplantation. The 11.0-Gy dose was delivered in two equal exposures administered 3 h apart. Mononuclear BM cells ($1-2 \times 10^5$) were injected into irradiated recipient mice.

NOS inhibitor administration. L-NAME (Sigma, St. Louis, MO, USA), D-NAME (Sigma), and ETU (Calbiochem, San Diego, CA, USA) were dissolved in 0.9% saline solution and sterilized by filtration. Animals were injected with 300 mg/kg body weight of L-NAME and 30 mg/kg body weight of ETU either directly or starting immediately after BM transplantation. In some experiments lower doses of inhibitors were used (Fig. 1c). Injections were performed intravenously or intraperitoneally twice a day for 3–17 days. The animals in the control group received injections of D-NAME (Fig. 1c) or saline solution.

Competitive repopulation assay. C57Bl/B6SjlPep3 (Ly-5.1) animals were treated with NOS inhibitors L-NAME and ETU or control solution either directly or after irradiation and BM transplantation. In the first setting, animals were treated for 6 or 12 days, and 0.5×10^5 BM cells were mixed with 2×10^5 Ly-5.2 competitor BM cells from C57Bl/6 mice and transplanted into the irradiated C57Bl/6 secondary recipients (Ly-5.2 genotype); 7 or 8 animals were used for each experimental point. In the second setting, animals were treated with inhibitors for 9 days after transplantation, and 1×10^5 or 5×10^5 BM cells were collected, mixed with 2×10^5 Ly-5.2 BM cells, and retransplanted into the irradiated C57Bl/6 secondary recipients; 8–12 animals were used for each experimental point. At various times (2 to 6 months for the first setting and 3 to 7 months for the second setting) after transplantation PB was collected from the retro-orbital venous plexus and analyzed by FACS for the relative contribution of donor (Ly-5.1) engraftment.

LTC-IC assay. Mice were treated with NOS inhibitors L-NAME and ETU or control solution for 9 days. BM cells were isolated from femurs of three to five mice and seeded in limiting dilutions (16 points per dilution) onto an irradiated BM stromal cell layer, prepared in advance. After 4 weeks cells were harvested and replated into methylcellulose in 35-mm dishes. All reagents for the analysis were purchased from StemCell Technologies and the assay was performed according to the manufacturer's instructions. After 12 days dishes were scored for the presence of colonies, and the stem cell equivalents per well were calculated using L-Calc software for the limiting dilution analysis (StemCell Technologies).

Pre-CFU-S assay. BM transplantation into primary recipients was performed as above and animals were treated with NOS inhibitors for 12 days. BM cells (1×10^5) or spleen cells (1×10^6) from 3–5 primary recipients were injected intravenously into 12–16 secondary irradiated recipient mice. Twelve days later their spleens were removed and fixed with Bouin's solution, and macroscopically visible spleen colonies were counted.

Colony forming assay. Mononuclear BM cells from the femurs of three mice were cultivated in 1% methylcellulose in Methocult GF M3434 medium (StemCell Technologies) at 2×10^4 per dish in triplicate. Colonies (>50 cells) were scored after 12 days.

Peripheral blood. Hematological analysis was performed using Hemavet 850 (Drew Scientific, Inc., Oxford, CT, USA). In some experiments erythrocytes and leukocytes were counted using a hemacytometer. Differential count of leukocytes was performed on smears stained with Hema 3 (Fisher). Hematocrit was determined using Micro-MB centrifuge (Thermo IEC, Needham Heights, MA, USA). Six to sixteen animals were used for each determination.

Flow cytometry analysis. BM cells were washed with PBS supplemented with 3% FBS, and erythrocytes were lysed with ammonium chloride–potassium bicarbonate buffer (Sigma). BM cells (1×10^6) were incubated with Sca-1, c-kit, or Sca-1/c-kit mAb for 30 min at 4°C. After washing, cells were treated with anti-CD16/CD32 mAb (Fc block) and stained for 30 min at 4°C with a cocktail of FITC-conjugated mAb against lineage markers (CD11b, CD5, CD45R, and Ly-6G). Cells were washed and fixed with 2% formaldehyde in PBS. Isotype-matched mAb were used for negative control. For competitive repopulation assay, PB cells were treated with ammonium chloride–potassium bicarbonate buffer and after being washed with PBS containing 2% FBS cells were stained with mAb against CD45.1 and CD45.2. Cells were washed and resuspended in PBS containing 2% FBS and 1 μg/ml propidium iodide. Samples were analyzed using an EPICS Elite flow cytometer (Beckman Coulter, Inc., Miami Lakes, FL, USA) or FACSCalibur (BD Biosciences, San Jose, CA, USA).

TUNEL assay. For the analysis of apoptosis, total mononuclear BM cells were fixed with 2% paraformaldehyde and permeabilized with ethanol, and apoptotic cells were labeled by TUNEL reaction using the *In Situ* Cell Death Detection Kit, Fluorescein (Roche Applied Science, Indianapolis, IN, USA). Samples were analyzed using an EPICS Elite flow cytometer. The histograms shown are representative examples of at least two independent experiments.

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Homing assay. BM cells were labeled with CFSE (Molecular Probes, Eugene, OR, USA) according to the manufacturer's instructions. Cells (20×10^6) were injected intravenously into lethally irradiated recipient mice, which then received intraperitoneal injections of NOS inhibitors or saline. BM cells were harvested 20 h after injection, treated with Fc block, and stained with mAb against Sca-1 and with StemSep murine hematopoietic progenitor enrichment cocktail of biotinylated mAb against lineage markers. After washing, the cells were stained with Streptavidin-PerCP and the number of $CFSE^+$ $Sca\text{-}1^+$ Lin^- cells was determined by FACS.

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