Published in final edited form as:

Vasc Med. 2010 October ; 15(5): 375–385. doi:10.1177/1358863X10378377.

Cholinergic activation of hematopoietic stem cells:

Role in tobacco-related disease?

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Abstract

Rationale—Tobacco use is associated with an increase in white blood cell count (WBC). This association has been attributed to bronchopulmonary inflammation and/or infection. It is not known if nicotine itself may play a role.

Objectives—We determined if nicotine itself could effect WBC count, and determined if this was due to a direct effect on hematopoietic stem cells (HSC).

Methods and Measurements—C57Bl6J mice received nicotine orally, and measurements of WBC count; bone marrow and spleen cellularity; and HSC count were made. To determine the functionality of HSCs, irradiated animals received bone marrow transplants from vehicle or nicotine treated mice.

Main Results—Nicotine increased leukocytes in the peripheral blood, bone marrow and spleen. Peripheral red cell and platelet count were unaffected. Nicotine increased the frequency of HSC in the bone marrow. Isolated long-term HSCs from nicotine-treated mice transplanted into irradiated mice regenerated all hematopoietic cell lineages, demonstrating functional competence of those HSCs. HSC expressed nicotinic acetylcholine receptors (nAChRs), as documented by FITC-conjugated alpha-bungarotoxin binding. Nicotine increased soluble Kit ligand, consistent with stem cell activation.

Conclusions—The data suggest a new mechanism for the increased WBC associated with tobacco use. The effect of nicotine to activate hematopoiesis may contribute to tobacco-related diseases.

Introduction

Tobacco use is associated with an increased white blood cell count^(1–3). This association may be of clinical significance as an increased white blood cell count is an independent risk factor for coronary heart disease and stroke.^{4–6,7} The increased WBC observed in smokers may not be entirely related to bronchopulmonary inflammation and/or infection.⁸

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Nevertheless, cigarette smoke contains constituents such as phenols (e.g. 3-methylphenol) and carbonyls (e.g. acrolein) that are known irritants of the bronchopulmonary mucosa. Notably, cigarette smoke activates neutrophils and increases their generation of superoxide anion.^{9,10}

Nicotine is a major active component of cigarette smoke and some adverse effects of smoking are attributable to this alkaloid.¹¹ Although most of the toxicity of tobacco exposure is related to other components, nicotine is responsible for the neurobehavioral effects and addiction.¹² Furthermore, nicotine stimulates the release of catecholamines, thereby increasing vascular tone, and inducing inotropic and chronotropic effects on the heart.¹³ These sympathomimetic effects have the untoward effect of increasing blood pressure and myocardial oxygen demand. Nicotine may also cause endothelial activation and vasodilator dysfunction, associated with the expression of adhesion molecules and chemokines that can facilitate vascular inflammation.^{14–16} Finally, nicotine can induce pathological angiogenesis.^{17,18} Whereas these effects might be anticipated to accelerate atherogenesis and precipitate major adverse cardiovascular events, short term use of nicotine patches has been shown to be safe in smokers with cardiovascular disease.¹⁹

More recently, non-neuronal nicotinic acetylcholine receptors (nAChRs) have been recognized to mediate some of the systemic effects of nicotine.^{14,17,20,21} The nAChRs are a class of ligand-gated ion channels that are variably permeable to sodium and calcium.²² Notably, circulating peripheral blood cells contain nAChRs.^{23,24} In the current study we tested the hypothesis that nicotine may directly affect WBC count.

Materials and Methods

Animals and treatment protocols

Female C57/BL6 mice were obtained from Jackson Laboratories and maintained by the Department of Laboratory Animals and Management at Stanford University. Mice were fed a normal chow diet (Purina) and given vehicle (0.4% saccharine in water) or nicotine solution (100 ug/ml nicotine in vehicle) to drink *ad libitum*. At 3, 6, 9, 14, 42 or 182 days after initiation of the study drug, groups of mice were sacrificed and the total cells from the bone marrow, spleen and periphery were isolated and prepared for assessment of cell number and composition. Unless otherwise stated, studies began with mice at 12 weeks of age.

Tissue and cell harvesting procedures

Peripheral blood cells—Mice were anaesthetized by intraperitoneal injection of xylazine/ketamine (16 and 80 mg/kg body weight respectively) and blood was obtained via cardiac puncture and collected into standard tubes (coated with EDTA as an anticoagulant) for complete blood cell counts (CBC) and fluorescence activated cell sorting (FACS) or flow cytometric analysis as described below.

Splenocytes—The spleen was harvested, fatty tissue carefully removed and the spleen weighed. The spleen was then pulverized in the presence of HBSS/2% FBS (staining buffer - SB) and strained through a 0.34 um filter mesh into a 50 ml Falcon tube. Total final volume was about 15 ml. An aliquot of cells (about 10⁶ cells) was processed for FACS analysis of B and T cells (CD8-FITC and CD4-PE and B220-Texas Red).

Bone marrow cells—Bone marrow cells were removed from the tibial and femoral bones of both hindlimbs by aspiration using an 18 gauge needle connected to a syringe containing 1ml of staining media in HBSS/2% FBS. Subsequently the cells were resuspended in 10ml

of HBSS/2% FBS, centrifuged (1000xg, 5 min, 4°C) and resuspended in 300 µl of ACK solution (a hypotonic solution consisting of 0.83% NH_2Cl /0.1% KHCO_3 in water) for 3–5 min to lyse red blood cells. To stop the lysis, the cells were resuspended in 10 volumes of HBSS/2% FBS. The cells were then centrifuged (1000xg, 5 min, 4°C), the supernatant removed and finally the cells were resuspended in 10 ml HBSS/2% FBS. The numbers of cells per ml were determined via standardized hemocytometric measurements and an aliquot of 1–2 million cells was reserved for flow cytometric analysis or sorting in the transplantation studies (see sections below). Cell suspensions (10 µl) were mixed with 190 µl Trypan blue. After 10 minutes of exposure, an aliquot of 15 µl was used for cell count using a standard hemocytometer and inverted light microscope.

Determination of erythrocyte count via hemoglobin content—We used hemoglobin content as a surrogate marker for bone marrow and splenic RBC count. We followed a modified protocol of the Drabkin assay.²⁵ Briefly, isolated bone marrow and splenic cells were centrifuged to a pellet (1000xg, 5 min, 4°C), the supernatant is drawn off and the cells resuspended in 500 µl of ACK solution for 5 minutes at 4°C. After 5 minutes an additional 500 µl of ACK solution is added to complete the lysis of rbc's. The cells are then centrifuged (1000xg, 5 min, 4°C), and the supernatant removed. To assay for hemoglobin content, one ml of the supernatant is mixed with 4 ml of a potassium ferricyanide/sodium azide mix known as Drabkin's reagent (Sigma, St. Louis, USA) and let stand in the dark for at least 15 minutes. The mixture is then assessed for absorbance at 540 nm. Actual hemoglobin content is then determined by comparison to a standard curve of dilutions (0–0.72 mg/ml protein) to bovine hemoglobin (Sigma, St. Louis, USA).

Flow cytometry

All flow cytometric analyses and fluorescence-activated cell sorting (FACS) were performed at the Shared FACS Facility at Stanford University using methods described previously.^{26,27} To detect myeloid cells we used the fluorescently labeled antibodies GR1-FITC and MAC1-PE. To detect B cells, B-220-Texas Red was used.

Hematopoietic stem cells (HSCs) were identified by negative staining for FITC-conjugated antibodies staining against the following lineage antigens: CD3, CD4, CD5, CD8 (T-cells), B220 (B-cells), Gr1 (Granulocytes), Mac1 (macrophages) and Ter 119 (erythrocytes) and positive antibody staining with anti-Sca1-Texas Red and anti-cKit-APC. Fluorescent antibodies to PE-conjugated Flk2 were used to differentiate short-term (ST, Flk2-positive) from long-term (LT, Flk2-negative) HSCs.²⁸ Myeloid progenitors were defined as c-kit⁺Lineage[−]Sca1[−] cells.

Functional Reconstitution of the bone marrow by LT-HSC transplantation

LT-HSC from 18-week old (control or nicotine-treated) mice (Ly5.1+ strain) were purified by FACS using the protocol described above.²⁸ Purified LT-HSC were transferred via retro-orbital venous injection into irradiated host mice (Ly5.2+ strain). The host mice were not treated with nicotine. Transfer of 25 or 100 cells was accompanied by 3×10^5 Sca-1 depleted bone marrow cells from the host strain. After 3 months, the mice were sacrificed, and blood cells analyzed as described above, to determine if there was donor-derived reconstitution.

FITC-alpha Bungarotoxin Binding

The snake venom peptide, alpha-bungarotoxin (abgt) is an irreversible antagonist with high affinity for the alpha7 and alpha 1 subunits of the nAChR, making it useful for identification of these receptor subunits.^{29–33} Total bone marrow cells or FACS sorted HSC were exposed to 10 µg/ml FITC-abgt (Sigma-Aldrich) for 2 hr at 20°C in HBSS/2% FBS. Control experiments to test for non-specific binding were performed by first adding 50-fold excess

of unlabelled abgt for 30 min at 20°C followed by 1.5 hr addition of FITC-abgt. FITC positive cells were visualized either through fluorescence microscopy on a Nikon TE-2000U or through FACS analysis.

Biochemical measurements

Plasma NOx was measured using the Griess reaction via a colorimetric assay (Cayman Chemicals). Plasma cotinine, a stable metabolite of nicotine, was measured by ELISA (OraSure Technologies). Plasma MMP-9 and soluble Kit ligand (SKitL) were measured by Quantikine ELISA (R&D Systems).

Statistical Analysis

Comparisons between control and nicotine-treated mice were analyzed either by Student's t-test (unpaired, two-sided) or 1-way ANOVA for experiments with more than two groups. One-way ANOVAs with post-hoc tests for linear trends were performed using GraphPad Prism version 4.00 for Windows. All data expressed as mean + SEM. Except where otherwise mentioned, the sample size, N, represents number of animals studied for both control and nicotine-treated mice. P-values of less than 0.05 were considered to be statistically significant.

Results

Nicotine alters white blood cell count

The administration of nicotine *ad libitum* in the drinking water resulted in plasma cotinine levels (40–80 ng/ml) that are similar to those in light to moderate smokers.³⁴ Plasma cotinine was undetectable in vehicle-treated animals. The administration of nicotine had no effect on red blood cell count (Figure 1a) mean corpuscular volume or mean corpuscular hematocrit count. Platelet count was also unaffected. By contrast, nicotine had striking effects on white blood cell count. After a transient decline at 3 days, there was a sustained increase in WBC count, peaking at 6 weeks (N=10 in each group, $p<0.01$, Figure 1a) was observed in nicotine treated mice. However, by 182 days, the WBC count was not different between nicotine-treated and control animals.

Nicotine increases bone marrow and spleen cellularity

In a second set of animals, the previous observations were confirmed. Absolute numbers of circulating wbcs for control vs. nicotine-treatment at 6 weeks were 4.6 ± 0.3 K/ul vs and 7.2 ± 0.7 K/ul (N=10, $p<0.01$, control vs. nicotine) respectively. The increase in cell count occurred across all leukocyte classes, and accordingly there was no alteration in the relative percentages of neutrophils, lymphocytes, and monocytes. Once again, we observed that nicotine did not change circulating RBC counts (9.7 ± 0.6 vs 8.9 ± 1.0 (M/ul) for control vs. nicotine treated mice respectively, N=10, NS).

To address the mechanisms of the increased WBC, we studied the cellularity and cell composition of the bone marrow and spleen in this set of animals. For these analyses, red blood cells were removed by lysis to focus on the leukocyte populations. Notably, nicotine caused changes in bone marrow and spleen cellularity that mirrored the temporal course of its effects on peripheral white blood cell counts (Figure 1b). Total cell counts in the bone marrow and spleen were transiently reduced at 3 days followed by an increase (Figure 1b). By six weeks of nicotine exposure, total cell counts in the bone marrow and spleen were significantly elevated (N=15 in each group; $p<0.005$ for bone marrow and $p<0.004$ for spleen; Figure 1b). Similarly, nicotine induced directionally and temporally similar changes in spleen weight. Nicotine administration caused a transient fall in spleen: body weight ratio at 3 days (0.33 ± 0.03 vs. 0.39 ± 0.01 ; Nicotine vs. Control; $p<0.001$; N=8 in each group),

which normalized at two weeks, (0.36 ± 0.02 vs. 0.38 ± 0.02 ; Nicotine vs. Control; $p = \text{NS}$; $N = 8$ in each group) progressing to a significant elevation in the ratio at 6 weeks (0.43 ± 0.02 vs. 0.38 ± 0.03 ; Nicotine vs. Control; $p < 0.001$; $N = 14$ in each group).

Mechanisms of nicotine-induced increase in WBC

In a separate series of studies, we confirmed the effects of nicotine to increase circulating wbc levels, and to increase bone marrow cellularity. At 6 weeks the increased white blood cell count was associated with increased bone marrow cellularity ($N = 15$ in each group; $p < 0.001$ versus control; Figure 1c). To determine if the increase in bone marrow or spleen cellularity was secondary to a specific leukocyte subpopulation, flow cytometric analysis was performed. The administration of nicotine did not induce a change in the frequency of neutrophils, B lymphocytes, T lymphocytes or monocytes in the bone marrow. Similar observations were made in the spleen, except for a slightly increased frequency of CD8 T cells (15% relative increase over control).

To determine if the increase in WBC and in cellularity of the bone marrow and spleen was associated with an amplification of HSCs, we performed flow cytometric analyses for HSCs. In the bone marrow, nicotine administration for six weeks increased HSC frequency 2.5 fold (Figure 2a). With G-CSF treatment, increase in HSC number is seen in the bone marrow within 2–3 days after exposure.³⁵ Nicotine, by way of contrast, did not significantly increase HSC pools by 3–14 days of exposure (data not shown). Although the Drabkin's assay detected higher levels of hemoglobin in the spleen of nicotine-treated animals at 6 weeks, we detected no significant increase in HSC in the spleen or blood at any time point. We next determined if there was an increase in the relative populations of long-term (LT-HSC) vs. short-term HSCs (ST-HSC). Both LT-HSC and ST-HSC subpopulations were increased in the nicotine treated animals at 6 weeks (Figure 2c and d).

We examined the relationship between total BM cell number and HSC number at 6 weeks of treatment with vehicle or nicotine. There was a positive correlation between the frequency of HSC and the total BM cell number (Figure 3a–b). A similar relationship between HSC frequency and spleen/body weight ratio was observed (data not shown). Flow cytometric analysis however yielded no significant change in HSCs in the spleen for nicotine-treated mice (HSCs in the spleen were infrequent in both groups, 10–20 HSCs/million cells). In preliminary studies, we found evidence for a nicotine-induced increase in myeloid progenitors. Specifically, after 6 weeks of treatment, nicotine-exposed mice ($n = 5$) manifested a 2-fold increase in the frequency ($p < 0.005$), and a 4-fold increase in the number ($p < 0.001$), of myeloid progenitor cells compared to vehicle-treated animals ($n = 5$).

Effects of nicotine on HSC engraftment

It is possible that nicotine would increase HSC number but not HSC function. Alternatively, a nicotine-induced increase in HSC proliferation could alter replicative capacity. To assess the function of HSCs, we isolated LT-HSCs (by FACS) from animals exposed to nicotine or vehicle for six weeks. The LT-HSCs were transplanted into lethally irradiated mice. Three months after transplantation of LT-HSCs, peripheral blood was obtained from the recipient mice and analyzed by FACS for B-cells (B220), T-cells (CD3), and macrophages (Mac1). Recipient mice displayed multilineage reconstitution of all major mononuclear cell types. Thus no significant differences in the ability of LT-HSCs from the nicotine treated animals to reconstitute the bone marrow (Figure 3c) were observed.

Expression of the nACh receptor by HSCs

It is possible that nicotine acts directly on HSCs through the nAChR. RT-PCR revealed detectable levels of $\alpha 7$ -nAChR subunit expression in the murine bone marrow (data not

shown) but not in the spleen. To determine the level and distribution of nAChR protein expression on HSC, we incubated whole bone marrow mononuclear cells (4a) or purified HSC (4b) with the high-affinity $\alpha 7$ -nAChR ligand, alpha bungarotoxin (abgt), conjugated to FITC and used FACS to detect for HSCs expressing nAChRs. The FACS data showed that a subpopulation of total bone marrow mononuclear cells expressed a receptor for abgt (4a). This binding was specific, as a 50-fold excess of unlabelled abgt abrogated the increase in fluorescence intensity observed with FITC-abgt (4a). Similarly, HSC (~40%) manifested specific FITC-abgt staining (Figure 4b).

Subsequently, we used FACS to isolate Sca1⁺ cells from bone marrow, and then stained this HSC-enriched fraction with FITC-labeled alpha-bungarotoxin.^{30,33} As shown in Figure 4c, many Sca1⁺ cells stained very faintly for alpha-bungarotoxin binding, with about 30% of cells staining strongly.

Nicotine increases plasma NO and SkitL

Plasma levels of nitric oxide and sKit ligand have been used as markers of stem cell activation.³⁶⁻⁴² At six weeks of nicotine administration, we observed increases in plasma levels of NOx (Figure 5a). Plasma sKitL levels were also elevated by nicotine (Figure 5b), within the range of reported levels for sKitL in the circulation (20–200 pg/ml).⁴³ Plasma levels of MMP-9, were not elevated (Figure 5c) by nicotine.

Discussion

The salient findings of this investigation are: 1) Systemic administration of nicotine increases the WBC; 2) this effect is associated with an increase in nucleated cells in the bone marrow and spleen, as well as 3) increased numbers of short and long-term hematopoietic stem cells, and 4) plasma markers of HSC mobilization. The HSCs from nicotine-treated animals are capable of engraftment and reconstitution of both myeloid and lymphoid cell lineages, indicating that the increase in HSC numbers does not alter replicative capacity. These data suggest the existence of novel pathway for hematopoiesis mediated by nicotinic acetylcholine receptors (nAChRs). This hypothesis is supported by the demonstration of nAChRs on a subpopulation of HSCs.

Our data are consistent with a previous report by Koval and colleagues.⁴⁴ They showed that in mice deficient for the $\alpha 7$ nAChR there was a reduction in myeloid and erythroid lineages. This latter finding is nearly the mirror image of our finding that nicotine-induced activation of nAChR in the bone marrow increases the proliferation of HSCs and increases the cellularity of the bone marrow. Notably, in $\alpha 7$ deficient mice, alpha-bungarotoxin binding disappeared from the bone marrow, confirming the specificity of the alpha-bungarotoxin for $\alpha 7$ nAChRs in the bone marrow cells. These data are consistent with our documentation of $\alpha 7$ nAChRs on HSCs.

Similarly, Pandit and colleagues provided evidence that nicotine could stimulate hematopoiesis.⁴⁵ Specifically, in BALB/c mice, a subcutaneous depot of nicotine, released over 21 days, increased hematopoiesis in the spleen (although had little effect on the bone marrow). In the spleen there was a striking increase in total colony formation as well as eosinophil-, granulocyte-macrophage-, and B-lymphocyte-specific progenitors. The major difference between our study, and that of Pandit et al.,⁴⁵ was the site of increased hematopoiesis induced by nicotine (spleen in Pandit et al, and bone marrow in our investigation). Perhaps this difference was related to the different strains of mice using in their study (BALB/c) versus ours (C57/Bl6).

We did find that nicotine increased spleen to body weight ratio, as well as hemoglobin content, phenomenon which could be due to extramedullary hyperplasia in the spleen and/or increased cellular sequestration. However we did not observe an increase in splenic HSCs with nicotine. In the absence of any increase in splenic hematopoietic stem cells, presumably the increased content of RBCs and WBCs in the spleen is due to sequestration in our model.

The nAChR is a ligand-gated calcium channel formed by a pentameric complex of nAChR subunits. The endogenous ligand is acetylcholine, which is a known neurotransmitter activating neuronal and neuromuscular nAChRs. We and others have described non-neuronal nAChRs that mediate other functions requiring intercellular communication including angiogenesis and inflammation.^{17,20,23,24} Nicotine hijacks this pathway to induce pathological neovascularization such as that observed in tumors, atherosclerotic plaque, or choroidal neovascularization.^{17,20} The current data provide a novel mechanism for another tobacco-related pathology, ie. an increased WBC. Smokers are known to have elevated white blood cell counts, which may be in part due to chronic irritation of the bronchopulmonary mucosa. In addition, smokers are at greater risk of bronchopulmonary infection. We have found evidence that nicotine may play a role in the increased WBC associated with tobacco.

In the first few days of nicotine exposure, we observed a transient reduction in WBC count in the periphery and bone marrow. We speculate that this early reduction in WBC counts in the peripheral blood and bone marrow may be due to sequestration of circulating leukocytes by the vascular endothelium and perhaps their infiltration into parenchymal tissues. Nicotine is known to enhance the adherence of hematopoietic stem/progenitor cells (HSPC) to the endothelium, an effect blocked by mecamylamine.⁴⁶ Furthermore, nicotine induces the expression of endothelial adhesion molecules and chemokines, and increases endothelial transmigration.⁴⁷ These actions of nicotine may have the effect of increasing WBC retention in peripheral tissues.

Smoking increases plasma fibrinogen levels and viscosity, as well as platelet count and hematocrit.⁴⁸ The erythrocytosis is believed to be secondary to an increase in carboxyhemoglobin, resulting in hypoxia-induced increases in erythropoietin. In our study, platelet and red blood cell counts in the circulation were not significantly affected. The lack of a comparable increase in peripheral rbc and platelet count suggests that precursor cells for these lineages are insensitive to nAChR stimulation, or their levels are more tightly (homeostatically) regulated; or they are sequestered in the spleen. In preliminary studies, we have observed evidence for the latter mechanism, ie. an increase in spleen and bone marrow hemoglobin content (a surrogate for erythrocyte mass). The effect of nicotine on erythropoiesis and thrombopoiesis requires further study.

Although the clinical literature supports a linkage between nicotine and the increased WBC associated with tobacco use,^{1,48} there are limited and conflicting mechanistic data. The existence of nACh receptors on leukocytes^{23,49} has been demonstrated. These receptors may mediate the effect of nicotine (*in vitro* exposure for 2 weeks) to suppress clonal expansion of hematopoietic and T-cell lineages of murine bone marrow isolates.⁵⁰⁻⁵² Furthermore, longer term exposure of nicotine (10^{-7} M and above) *in vitro* significantly down-regulates several nicotinic acetylcholinergic (nAChR) receptor subunits in leukemic T-cell lines.⁵⁰ By contrast, *in vivo* studies found that chronic exposure to nicotine increased the surface density of select subunits of nAChR in peripheral mononuclear cells.^{23,24} The discordance in these observations may be related in part to differences in the duration and dose of nicotine exposure. Indeed, in another model system (endothelial cell proliferation and angiogenesis) nicotine has biphasic effects that are dose- and time-dependent.^{20,53} Indeed, long-term

administration of nicotine downregulates vascular $\alpha 7$ nAChRs and impairs angiogenesis.⁵⁴

In this regard, chronic exposure to nicotine may adversely affect hematopoiesis. Koval and colleagues⁽⁴⁴⁾ found that after long-term exposure to oral nicotine, there was a reduction in the murine bone marrow of myeloblasts, promyelocytes and erythroblasts. This apparent discordance with our data is explained by the fact that they exposed mice to nicotine in the drinking water at a concentration of 200ug/ml for 10 months, which was twice as much nicotine for almost twice as long as the studies carried out by us. They suggested that their results were due to receptor desensitization of $\alpha 7$ -containing nicotinic receptors which play a major role in regulating hematopoiesis. Of note, in our study, the effect of nicotine to increase the WBC count seemed to diminish between days 42 to 182. So in our study, even at a lower dose of oral nicotine, we may have begun to induce some nAChR desensitization.

The response of the hematopoietic system to nicotine may be different during development. Seroby et al studied the effects of nicotine on the murine fetal bone marrow.⁵⁵ They provided evidence that nicotine impaired colonization of fetal bone marrow by HSPC immigrating from the fetal liver, thereby reducing the number of mature lymphocytes, and the strength of the immune response in neonates. Differences between our work and theirs are likely due the models (adult versus fetal mice) and/or the mode of nicotine administration (intravenous versus oral). Notably, nicotine accumulates in fetal tissues, “likely due to immature enzymatic activity in the placenta and fetal liver”, to quote Seroby et al and co-workers.⁵⁵

In any event, our studies indicate that nicotine may have direct effects on hematopoietic stem cell proliferation and activation. Stem cells have both differentiation and self-renewal capacities. Short-term HSCs (which are flk2⁺, KLS cells) have a limited capacity for self-renewal, whereas long-term HSCs (flk2⁻, KLS cells) have an indefinite capacity for self-renewal. Both LT-HSC and ST-HSC may differentiate into any of the leukocyte lineages. We found that nicotine increased the number of both subtypes of HSCs as well as numbers of each lymphoid and myeloid cell lines in a sustained manner. The functionality of the long-term HSCs in the nicotine-treated animals was confirmed by documenting their ability to establish multilineage reconstitution of leukocyte lineages in lethally irradiated mice.

Indeed, the reconstitution levels upon transplantation of nicotine-treated HSC tended to be greater than those from vehicle-treated animals. This observation is consistent with our data showing that nicotine treatment increases HSC (Figure 2) and WBC (Figure 1) numbers and may indicate a proliferative effect of nicotine on HSC, ie. there may be an enhancement of HSC engraftment and/or regeneration of the hematopoietic system with stimulation of the HSC nAChR.

Although the effects of nicotine on the bone marrow are similar to those of granulocyte colony stimulating factor (G-CSF), there are important differences. The effects of G-CSF on mobilization and proliferation of HSCs, with attendant leukocytosis, occur within 2–3 days³⁵ and immature HSCs can appear in the circulation. Like G-CSF, nicotine increases plasma levels of nitric oxide and sKit ligand, biomarkers of stem cell mobilization,^{36–42,56} Nitric oxide is believed to nitrosylate and thereby activate matrix metalloproteinases, which then cleave the tethered ligand cKitL.⁵⁷ This produces sKitL and releases HSCs from their niche, potentially inducing their proliferation and migration. We did not observe an increase in plasma levels of MMP-9, but we did not test for an increase in expression or activation of MMP-9 locally in the bone marrow.

Nicotine recruits circulating endothelial precursors to sites of tumor neovascularization in murine models.⁵⁸ This is consistent with previous observations that nicotine can induce the

release of vascular endothelial growth factor as well as fibroblast growth factor from endothelial cells, and that systemic administration of nicotine can increase circulating levels of these growth factors.^{59, 60} Furthermore, it is likely that bone marrow derived endothelial progenitor cells share a common precursor with hematopoietic stem cells.^{56,61} In explanation of our data and that of Natori and colleagues,⁵⁸ there may be a common precursor for endothelial cells and leukocytes that is responsive to stimulation by endogenous acetylcholine or nicotine. Indeed, we found PCR, FACS and immunohistochemical evidence for nACh receptors on a subpopulation of HSCs. The presence of alpha-bungarotoxin binding sites on 20–40% of the HSCs suggests an important role for endogenous cholinergic regulation of stem cell activation and proliferation.

Exogenous nicotine may hijack this pathway to stimulate HSC activation, proliferation, and differentiation. If so, the question arises as to whether chronic stimulation by nicotine (as with smokers) could induce a “replicative exhaustion”. Indeed, in chronic smokers, circulating endothelial progenitor cells are reduced, and tobacco cessation improves the numbers of circulating EPCs.⁶² However, we did not observe a decline in the numbers of long-term HSCs, nor their functionality after six weeks of nicotine exposure. More chronic exposure to nicotine could ultimately induce a “replicative exhaustion”. Indeed, it could be argued that there is subtle evidence within our data for an early nicotine-induced decline in replicative capacity. Figure 3 shows that, when both nicotine-treated and control animals are considered, there is a positive correlation between the frequency and number of HSCs, and the cellularity of the bone marrow. This is not an unexpected finding. However, the slope of this correlation tends to be shallower when considering only the nicotine-treated animals. This observation could reflect a decline in the replicative capacity in the HSC from nicotine-treated mice. Another possibility is that the nicotine-induced increase in HSC may saturate the limited number of HSC niches in the bone marrow thereby rendering a more shallow correlation between HSC frequency and total bone marrow number. The possibility that stem cell niches in the bone marrow may become saturated has experimental support.^{63,64}

In summary, we find that chronic exposure to nicotine induces an increase in WBC and in cellularity of the bone marrow and spleen. All white blood cell lineages seem equally affected. These effects are associated with an increase in hematopoietic stem cells in the bone marrow. A subpopulation of the HSCs expresses the nACh receptor. Nicotine may exert its effects directly through this receptor, and/or by activating mediators of HSC mobilization. These findings suggest a novel mechanism for the increased WBC associated with tobacco, and suggest a role for cholinergic regulation of hematopoiesis.

Acknowledgments

This study was supported by grants from the National Institutes of Health (RO1 HL-75774, RO1 CA098303, R21 HL085743, RC2HL103400, 1U01HL100397), the Tobacco Related Disease Research Program of the University of California (18XT-0098), Philip Morris USA Inc, and by the Cancer Research Institute (for ECF). Dr. Cooke is an inventor on Stanford University patents related to therapeutic modulation of angiogenesis by agonists or inhibitors of the nACh receptors. These patents have been licensed to Comentis Inc., in which company Dr. Cooke has equity.

Abbreviations

abgt	Alpha-bungarotoxin
BMC	Bone Marrow Cell
HSC	Hematopoietic Stem Cells
ST-HSC	Short-term hematopoietic stem cells

LT-HSC	Long Term Hematopoietic Stem Cells
nAChR	Nicotinic Acetylcholine Receptor
NO	Nitric Oxide
cKit	cKit Receptor
SKitL	soluble Kit ligand
Sca1	Stem cell antigen 1
rbc	Red blood cells or erythrocytes
wbc	Peripheral white blood cells
KLS	cKit ⁺ /lin ⁻ /Sca1 ⁺ HSCs

References

1. Jensen EJ, Pedersen B, Frederiksen R, Dahl R. Prospective study on the effect of smoking and nicotine substitution on leucocyte blood counts and relation between blood leucocytes and lung function. *Thorax*. 1998; 53:784–789. [PubMed: 10319062]
2. Corre F, Lellouch J, Schwartz D. Smoking and leucocyte-counts. Results of an epidemiological survey. *Lancet*. 1971; 2:632–634. [PubMed: 4105947]
3. Sparrow D, Glynn RJ, Cohen M. The relationship of the peripheral leukocyte count and cigarette smoking to pulmonary function among adult men. *Chest*. 1984; 86:383–386. [PubMed: 6467999]
4. Ensrud K, Grimm RHJ. The white blood cell count and risk for coronary heart disease. *Am Heart J*. 1992; 124:207–213. [PubMed: 1615807]
5. Madjid M, Awan I, Willerson JT, Casscells SW. Leukocyte count and coronary heart disease: implications for risk assessment. *J Am Coll Cardiol*. 2004; 44:1945–1956. [PubMed: 15542275]
6. Haim M, Boyko V, Goldbourt U, Battler A, Behar S. Predictive value of elevated white blood cell count in patients with preexisting coronary heart disease: the Bezafibrate Infarction Prevention Study. *Arch Intern Med*. 2004; 164:433–439. [PubMed: 14980995]
7. Friedman GD, Klatsky AL, Siegelaub AB. The leukocyte count as a predictor of myocardial infarction. *N Engl J Med*. 1974; 290:1275–1278. [PubMed: 4827627]
8. Friedman GD, Siegelaub AB, Seltzer CC, Feldman R, Collen MF. Smoking habits and the leukocyte count. *Arch Environ Health*. 1973; 26:137–143. [PubMed: 4688852]
9. Pitzer JE, Del Zoppo GJ, Schmid-Schonbein GW. Neutrophil activation in smokers. *Biorheology*. 1996; 33:45–58. [PubMed: 8869343]
10. Koethe SM, Kuhnmuench JR, Becker CG. Neutrophil priming by cigarette smoke condensate and a tobacco anti-idiotypic antibody. *Am J Pathol*. 2000; 157:1735–1743. [PubMed: 11073832]
11. Calafat AM, Polzin GM, Saylor J, Richter P, Ashley DL, Watson CH. Determination of tar, nicotine, and carbon monoxide yields in the mainstream smoke of selected international cigarettes. *Tob Control*. 2004; 13:45–51. [PubMed: 14985595]
12. Benowitz NL, Hukkanen J, Jacob P 3rd. Nicotine chemistry, metabolism, kinetics and biomarkers. *Handb Exp Pharmacol*. 2009;29–60. [PubMed: 19184645]
13. Benowitz NL. Cigarette smoking and cardiovascular disease: pathophysiology and implications for treatment. *Prog Cardiovasc Dis*. 2003; 46:91–111. [PubMed: 12920702]
14. Zhu BQ, Heeschen C, Sievers RE, Karliner JS, Parmley WW, Glantz SA, Cooke JP. Second hand smoke stimulates tumor angiogenesis and growth. *Cancer Cell*. 2003; 4:191–196. [PubMed: 14522253]
15. Heeschen C, Jang J, Hoai-Ky V, Kaji S, Yang P, HR, JPC. Nicotine is an agent of angiogenesis. *Nature Medicine*. 2001; 7:833–839.
16. Puranik R, Celermajer DS. Smoking and endothelial function. *Prog Cardiovasc Dis*. 2003; 45:443–458. [PubMed: 12800127]

17. Heesch C, Jang JJ, Weis M, Pathak A, Kaji S, Hu RS, Tsao PS, Johnson FL, Cooke JP. Nicotine stimulates angiogenesis and promotes tumor growth and atherosclerosis. *Nat Med.* 2001; 7:833–839. [PubMed: 11433349]
18. Heesch C, Weis M, Aicher A, Dimmeler S, Cooke J. A novel angiogenic pathway mediated by non-neuronal nicotinic acetylcholine receptors. *J Clin Invest.* 2002; 110:527 – 536. [PubMed: 12189247]
19. Joseph AM, Norman SM, Ferry LH, Prochazka AV, Westman EC, Steele BG, Sherman SE, Cleveland M, Antonuccio DO, Hartman N, McGovern PG. The safety of transdermal nicotine as an aid to smoking cessation in patients with cardiac disease. *N Engl J Med.* 1996; 335:1792–1798. [PubMed: 8943160]
20. Heesch C, Weis M, Aicher A, Dimmeler S, Cooke JP. A novel angiogenic pathway mediated by non-neuronal nicotinic acetylcholine receptors. *J Clin Invest.* 2002; 110:527–536. [PubMed: 12189247]
21. Sharma G, Vijayaraghavan S. Nicotinic receptor signaling in nonexcitable cells. *J Neurobiol.* 2002; 53:524–534. [PubMed: 12436417]
22. Gotti C, Clementi F. Neuronal nicotinic receptors: from structure to pathology. *Prog Neurobiol.* 2004; 74:363–396. [PubMed: 15649582]
23. Benhammou K, Lee M, Strook M, Sullivan B, Logel J, Raschen K, Gotti C, Leonard S. [(3)H]Nicotine binding in peripheral blood cells of smokers is correlated with the number of cigarettes smoked per day. *Neuropharmacology.* 2000; 39:2818–2829. [PubMed: 11044752]
24. Lebargy F, Benhammou K, Morin D, Zini R, Urien S, Bree F, Bignon J, Branellec A, Lagrue G. Tobacco smoking induces expression of very-high-affinity nicotine binding sites on blood polymorphonuclear cells. *Am J Respir Crit Care Med.* 1996; 153:1056–1063. [PubMed: 8630545]
25. Moore GL, Ledford ME, Merydith A. A micromodification of the Drabkin hemoglobin assay for measuring plasma hemoglobin in the range of 5 to 2000 mg/dl. *Biochem Med.* 1981; 26:167–173. [PubMed: 7317038]
26. Forsberg EC, Serwold T, Kogan S, Weissman IL, Passegue E. New evidence supporting megakaryocyte-erythrocyte potential of flk2/flt3+ multipotent hematopoietic progenitors. *Cell.* 2006; 126:415–426. [PubMed: 16873070]
27. Forsberg EC, Prohaska SS, Katzman S, Heffner GC, Stuart JM, Weissman IL. Differential expression of novel potential regulators in hematopoietic stem cells. *PLoS Genet.* 2005; 1:e28. [PubMed: 16151515]
28. Christensen JL, Weissman IL. Flk-2 is a marker in hematopoietic stem cell differentiation: a simple method to isolate long-term stem cells. *Proc Natl Acad Sci U S A.* 2001; 98:14541–14546. [PubMed: 11724967]
29. Baum CM, Weissman IL, Tsukamoto AS, Buckle AM, Peault B. Isolation of a candidate human hematopoietic stem-cell population. *Proc Natl Acad Sci U S A.* 1992; 89:2804–2808. [PubMed: 1372992]
30. Sabban EL, Gueorguiev VD. Effects of short- and long-term nicotine treatment on intracellular calcium and tyrosine hydroxylase gene expression. *Ann N Y Acad Sci.* 2002; 971:39–44. [PubMed: 12438086]
31. Spangrude GJ, Heimfeld S, Weissman IL. Purification and characterization of mouse hematopoietic stem cells. *Science.* 1988; 241:58–62. [PubMed: 2898810]
32. Spangrude GJ, Smith L, Uchida N, Ikuta K, Heimfeld S, Friedman J, Weissman IL. Mouse hematopoietic stem cells. *Blood.* 1991; 78:1395–1402. [PubMed: 1884012]
33. Wang Y, Pereira EFR, Maus ADJ, Ostlie NS, Navaneetham D, Lei S, Albuquerque EX, Conti-Fine BM. Human Bronchial Epithelial and Endothelial Cells Express alpha-7 Nicotinic Acetylcholine Receptors. *Molecular Pharmacology.* 2001; 60:1201–1209. [PubMed: 11723227]
34. Benowitz NL, Zevin S, Jacob Pr. Sources of variability in nicotine and cotinine levels with use of nicotine nasal spray, transdermal nicotine, and cigarette smoking. *Br J Clin Pharmacol.* 1997; 43:259–267. [PubMed: 9088580]
35. Seggewiss R, Buss EC, Herrmann D, Goldschmidt H, Ho AD, Fruehauf S. Kinetics of peripheral blood stem cell mobilization following G-CSF-supported chemotherapy. *Stem Cells.* 2003; 21:568–574. [PubMed: 12968111]

36. Ziche M, Morbidelli L, Choudhuri R, Zhang HT, Donnini S, Granger HJ, Bicknell R. Nitric oxide synthase lies downstream from vascular endothelial growth factor-induced but not basic fibroblast growth factor-induced angiogenesis. *J Clin Invest.* 1997; 99:2625–2634. [PubMed: 9169492]
37. Ziche M. Nitric Oxide promotes proliferation and plasminogen activator production by coronary venular endothelium through endogenous. *Circ Res.* 1993; 80:845–852. [PubMed: 9168787]
38. Witzenbichler B, Asahara T, Murohara T, Silver M, Spyridopoulos I, Magner M, Principe N, Kearney M, Hu JS, Isner JM. Vascular endothelial growth factor-C (VEGF-C/VEGF-2) promotes angiogenesis in the setting of tissue ischemia. *Am J Pathol.* 1998; 153:381–394. [PubMed: 9708799]
39. Noiri E, Lee E, Testa J, Quigley JP, Colflesh D, Keese CR, Giaever I, Goligorsky MS. Podokinesis in endothelial cell migration: role of nitric oxide. *Am J Physiol.* 1998; 274:C236–C244. [PubMed: 9458733]
40. Morbidelli L, Chang CH, Douglas JG, Granger HJ, Ledda F, Ziche M. Nitric oxide mediates mitogenic effect of VEGF on coronary venular endothelium. *Am J Physiol.* 1996; 270:H411–415. [PubMed: 8769777]
41. Lyden D, Hattori K, Dias S, Costa C, Blaikie P, Butros L, Chadburn A, Heissig B, Marks W, Witte L, Wu Y, Hicklin D, Zhu Z, Hackett NR, Crystal RG, Moore MA, Hajjar KA, Manova K, Benezra R, Rafii S. Impaired recruitment of bone-marrow-derived endothelial and hematopoietic precursor cells blocks tumor angiogenesis and growth. *Nat Med.* 2001; 7:1194–1201. [PubMed: 11689883]
42. Inoue N, Venema RC, Sayegh HS, Ohara Y, Murphy TJ, Harrison DG. Molecular regulation of the bovine endothelial cell nitric oxide synthase by transforming growth factor-beta 1. *Arterioscler Thromb Vasc Biol.* 1995; 15:1255–1261. [PubMed: 7543000]
43. Heissig B, Hattori K, Dias S, Friedrich M, Ferris B, Hackett N, Crystal R, Besmer P, Lyden D, Moore M, Werb Z, Rafii S. Recruitment of stem and progenitor cells from the bone marrow niche requires MMP-9 mediated release of kit-ligand. *Cell.* 2002; 109:625 – 637. [PubMed: 12062105]
44. Koval LM, Zverkova AS, Grailhe R, Utkin YN, Tsetlin VI, Komisarenko SV, Skok MV. Nicotinic acetylcholine receptors alpha4beta2 and alpha7 regulate myelo- and erythropoiesis within the bone marrow. *Int J Biochem Cell Biol.* 2008; 40:980–990. [PubMed: 18083057]
45. Pandit TS, Sikora L, Muralidhar G, Rao SP, Sriramara P. Sustained exposure to nicotine leads to extramedullary hematopoiesis in the spleen. *Stem Cells.* 2006; 24:2373–2381. [PubMed: 16825610]
46. Seroby N, Schraufstatter IU, Strongin A, Khaldoyanidi SK. Nicotinic acetylcholine receptor-mediated stimulation of endothelial cells results in the arrest of haematopoietic progenitor cells on endothelium. *Br J Haematol.* 2005; 129:257–265. [PubMed: 15813854]
47. Heeschen C, Chang E, Johnson FL, Pathak-Gulati A, Aicher A, Dimmeler S, Cooke JP. Stimulation of Nicotinic Acetylcholine Receptors Promotes Recruitment of Endothelial Progenitor Cells During Ischemia. *Circ Res.* 2005 submitted.
48. Ernst E. Haemorheological consequences of chronic cigarette smoking. *J Cardiovasc Risk.* 1995; 2:435–439. [PubMed: 8749271]
49. Cormier A, Paas Y, Zini R, Tillement JP, Lagrue G, Changeux JP, Grailhe R. Long-term exposure to nicotine modulates the level and activity of acetylcholine receptors in white blood cells of smokers and model mice. *Mol Pharmacol.* 2004; 66:1712–1718. [PubMed: 15383622]
50. Kimura R, Ushiyama N, Fujii T, Kawashima K. Nicotine-induced Ca²⁺ signaling and down-regulation of nicotinic acetylcholine receptor subunit alpha-expression in the CEM human leukemic T-cell line. *Life Sci.* 2003; 72:2155–2158. [PubMed: 12628473]
51. Khaldoyanidi S, Sikora L, Orlovskaya I, Matrosova V, Kozlov V, Sriramara P. Correlation between nicotine-induced inhibition of hematopoiesis and decreased CD44 expression on bone marrow stromal cells. *Blood.* 2001; 98:303–312. [PubMed: 11435297]
52. Takahashi M, Shigeno N, Narita M, Takahashi H, Furukawa T, Koike T, Aizawa Y. Cotinine (a metabolite of nicotine) suppresses the growth of hematopoietic progenitor cells at the concentration range equivalent to its serum levels in smokers. *Stem Cells.* 1999; 17:125–126. [PubMed: 10195574]
53. Villablanca AC. Nicotine stimulates DNA synthesis and proliferation in vascular endothelial cells in vitro. *J Appl Physiol.* 1998; 84:2089–2098. [PubMed: 9609804]

54. Konishi H, Wu J, Cooke JP. Chronic exposure to nicotine impairs cholinergic angiogenesis. *Vasc Med.* 2010; 15:47–54. [PubMed: 19778953]
55. Serobyan N, Jagannathan S, Orlovskaya I, Schraufstatter I, Skok M, Loring J, Khaldoyanidi S. The cholinergic system is involved in regulation of the development of the hematopoietic system. *Life Sci.* 2007; 80:2352–2360. [PubMed: 17512954]
56. Rafii S, Lyden D. Therapeutic stem and progenitor cell transplantation for organ vascularization and regeneration. *Nat Med.* 2003; 9:702–712. [PubMed: 12778169]
57. Aicher A, Heeschen C, Mildner-Rihm C, Urbich C, Ihling C, Technau-Ihling K, Zeiher AM, Dimmeler S. Essential role of endothelial nitric oxide synthase for mobilization of stem and progenitor cells. *Nat Med.* 2003; 9:1370–1376. [PubMed: 14556003]
58. Natori T, Sata M, Washida M, Hirata Y, Nagai R, Makuuchi M. Nicotine enhances neovascularization and promotes tumor growth. *Mol Cells.* 2003; 16:143–146. [PubMed: 14651253]
59. Carty CS, Soloway PD, Kayastha S, Bauer J, Marsan B, Ricotta JJ, MD. Nicotine and cotinine stimulate secretion of basic fibroblast growth factor and affect expression of matrix metalloproteinases in cultured human smooth muscle cells. *J Vasc Surg.* 1996; 24:927–934. [PubMed: 8976346]
60. Conklin BS, Zhao W, Zhong DS, Chen C. Nicotine and cotinine up-regulate vascular endothelial growth factor expression in endothelial cells. *Am J Pathol.* 2002; 160:413–408. [PubMed: 11839560]
61. Pelosi E, Valtieri M, Coppola S, Botta R, Gabbianelli M, Lulli V, Marziali G, Masella B, Muller R, Sgadari C, Testa U, Bonanno G, Peschle C. Identification of the hemangioblast in postnatal life. *Blood.* 2002; 100:3203–3208. [PubMed: 12384418]
62. Kondo T, Hayashi M, Takeshita K, Numaguchi Y, Kobayashi K. Smoking cessation rapidly increases circulating progenitor cells in peripheral blood in chronic smokers. *Arterioscler Thromb Vasc Biol.* 2004; 24:1442–1447. [PubMed: 15191940]
63. Czechowicz A, Kraft D, Weissman IL, Bhattacharya D. Efficient transplantation via antibody-based clearance of hematopoietic stem cell niches. *Science.* 2007; 318:1296–1299. [PubMed: 18033883]
64. Wilson A, Trumpp A. Bone-marrow haematopoietic-stem-cell niches. *Nat Rev Immunol.* 2006; 6:93–106. [PubMed: 16491134]

Figure 1a:

Figure 1b

Figure 1c

Figure 1.

(A) Time course of peripheral rbc and wbc counts as a function of nicotine administration. Values are comparative population ratios for peripheral cell populations of nicotine-treated vs. control mice and were obtained by setting rbc and wbc values for control mice at 100 and normalizing the values for the nicotine-treated mice around this reference point. Typical rbc and wbc values for control C57Bl6 mice are 9.8 ± 0.8 (M/ul) and 2.1 ± 0.2 (K/ul) respectively. Samples sizes per experimental groups are: N=5 for 3 days, N=10 for 14 and 42 days, N=3 for 184 days. (B) Nicotine induces a time dependent alteration in bone marrow and splenic cellularity in C57Bl6 mice. Values represent % population ratios of nicotine-treated over control animals. 1-way ANOVA revealed a significant linear trend for both bone marrow and spleen as a function of nicotine exposure. ($P < 0.001$; N=4–10 per group depending upon the time point). A * represents $P < 0.01$ vs. control values (Student t-test). (C) The effect of nicotine at six weeks on absolute cell number of nucleated cells in bone marrow and spleen.

Figure 2.

A 6-week nicotine exposure to C57Bl6 mice increases (A) HSC frequency and (B) total HSC (C) LT-HSC and (D) ST-HSC counts in the bone marrow when compared to vehicle treated mice (N=19 for control group, N=21 for nicotine group). Values are expressed as %HSC frequency in the bone marrow for 2A, whereas values in Figures 2B–D represent HSCs per two femurs. In C57Bl/6 mice, the normal range for LT-HSC is 2.4 ± 0.06 LT-HSC/ 10^5 bone marrow mononuclear cells (Muller-Sieburg and Riblet, 1996). Mean plasma cotinine level in nicotine-treated mice was 87 ng/ml, similar to that in smoking humans.

Figure 3.

(A–B) Positive correlation between HSC frequency in bone marrow and total nucleated cell content for control (open triangle) and nicotine-treated (closed squares) mice ($P < 0.001$ for both groups). We examined the relationship between total BM cell number and HSC number at 6 weeks of treatment with vehicle or nicotine. Scatter plots expressed as: (A) % HSC frequency vs. total bone marrow cell count per 2 femur or (B) Total HSC cells vs. total bone marrow cell count per 2 femur ($N = 19$ for control group, $N = 21$ for nicotine group). In both Figure 3A and 3B the slopes for the nicotine-treated samples were significantly different from their equivalent controls by at least $P < 0.01$. For Figure 3A, slopes and R^2 values for control vs. nicotine samples are 384 and 0.77 vs. 182 and 0.49 respectively. In Figure 3B, the equivalent parameters for control vs. nicotine are: 0.001 and 0.80 vs. 0.0005 and 0.72 respectively. (C) LT-HSC transplantation of Ly5.1 positive cells into irradiated Ly5.2 positive murine hosts results in functional reconstitution of peripheral blood cells in the circulation. The light bars represent cells originating from the HSC from vehicle-treated animals while the dark bars represent data for cells originating from HSC of nicotine-treated mice. Data expressed as % chimerism of indicated cell type in periphery (i.e. percent of cell type that originates from donor LT-HSC). Symbols: Multi-Lineage is total Ly5.1 positive cells, B220-marker for B-type cells, CD3-marker for T-Cells, Mac1- marker for macrophages. Sample sizes are: Nicotine, $N = 14$, Control $N = 9$. Data represents results from an initial input of 100 LT-HSC into each recipient mouse.

(a)

(b)

Figure 4c

Figure 4.

Representative flow cytometry analysis showing binding of FITC-alpha-bungarotoxin to total mononuclear bone marrow cells (A) and to HSCs (B). Fluorescent micrographs of (C) Alpha-Bungarotoxin binding to Sca1⁺ cells from murine mononuclear bone marrow cells.

Figure 5.

(A) Plasma NOx levels in nicotine-treated mice (6 weeks exposure). N=5 per experimental group. (B) Plasma SKitL levels in control mice and in mice after a 6-week treatment with nicotine. N=5 per experimental group. (C) Plasma MMP9 levels in control and nicotine-treated mice. N=5 per experimental group