NICOTINIC REGULATION OF HEMATOPOIESIS

BY

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THESIS

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Abstract

Chronic activation of the sympathetic nervous system (SNS) contributes to increased myelopoiesis. Since nicotine activates the SNS, chronic nicotine consumption may also increase myelopoiesis. This is relevant to human health outcomes because myelopoiesis contributes to the progression of atherosclerosis and cardiovascular disease. To address this, mice were provided high dose nicotine in drinking water for two and three weeks, and leukocyte production and distribution were assessed. Nicotine supplementation for two weeks did not alter the production or distribution of leukocytes, but three weeks of nicotine increased hematopoietic progenitor proliferation in the spleen and mildly increased leukocyte counts in the spleen but not blood. Next, the sympathomimetic drug guanethidine was used to assess the effect of SNS inhibition during nicotine exposure. In this study, guanethidine did not influence leukocyte distribution or production in mice treated with nicotine. Collectively, three weeks of nicotine supplementation enhanced splenic hematopoiesis without meaningfully increasing the systemic leukocyte supply.

Acknowledgments

«El recuerdo que deja un libro es más importante que el libro mismo.»

Gustavo Adolfo Bécquer

I am very grateful with the people that have made this experience possible. I would like to express my gratitude to professors, peers, and friends, which have been there during this process; their support has been key and highly valued.

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Chapter 1: Introduction

Chronic tobacco smoking increases the risk of atherosclerosis and cardiovascular disease (Benowitz et al., 1997), but the underlying biological mechanisms are still debated or unknown. Relevant to this, enhanced myelopoiesis is increasingly recognized as a common mechanism connecting multiple cardiovascular disease risk factors to accelerated atherosclerosis, due to the contribution of immune cells during plaque formation. For instance, hypercholesteremia (Yvan-Charvet et al., 2010), obesity (Nagareddy et al., 2013), hyperglycemia (Flynn et al., 2020; Nagareddy et al., 2013), and chronic psychological stress (Heidt et al., 2014; Tawakol et al., 2017) all enhance the production of myeloid cells that promote atherosclerosis. Enhanced myelopoiesis during these conditions accelerates the accumulation of monocytes to early atherosclerotic plaques that differentiate into dysfunctional and inflammatory foam cells (Swirski et al., 2007). In this way, enhanced myelopoiesis represents an important mechanism connecting these risk factors to cardiovascular disease. In particular, chronic psychological stress accelerates atherosclerosis via SNS-dependent enhancement of myelopoiesis (Heidt et al., 2014). Since nicotine also activates the SNS (Haass & Kubler, 1996), then it may induce myelopoiesis in a similar SNS-dependent manner.

Despite a conspicuous sparsity if reports on the subject, some have reported that smoking tobacco is associated with increased myeloid cells in circulation (Andreoli et al., 2015; Jensen et al., 1998). However, it is unclear if the effects of smoking on cardiovascular disease and myeloid counts are caused by nicotine or if they are caused by other particulates produced by combustion during smoking. Moreover, the preclinical literature has thoroughly addressed the notion that nicotine accelerates atherosclerosis, but the effect of chronic nicotine exposure on myelopoiesis

has not clearly been addressed (Centner et al., 2020; Lee & Cooke, 2011). These collective observations highlight the possibility that nicotine may contribute to atherosclerosis via myelopoiesis. For this reason, it is important to experimentally address the myelopoietic effects of nicotine consumption in the absence potential confounders, such as tobacco combustion.

Nicotine stimulates nicotinic acetylcholine receptors on postganglionic sympathetic neurons (Haass & Kubler, 1996). This results in depolarization and the release of NE from postganglionic neurons into tissues (Haass & Kubler, 1996). The hematopoietic niches in the spleen and bone marrow are innervated by noradrenergic postganglionic cells from the SNS that influence the activity and differentiation of hematopoietic progenitors both directly and indirectly through stromal cells (Agarwala & Tamplin, 2018; Aguila, 2006). Both hematopoietic stem cells (HSCs) and the immunoregulatory mesenchymal stromal cells (MSCs) express beta adrenergic receptors (Méndez-Ferrer, Michurina, et al., 2010). Sympathetic innervation of hematopoietic niches is important for the maintenance of normal hematopoietic physiology (Katayama et al., 2006). Activation of β2 and β3 adrenergic receptors reduces CXCL12 expression on mesenchymal bone marrow cells and increases activity of HSCs (Katayama et al., 2006; Méndez-Ferrer et al., 2008; Méndez-Ferrer, Battista, et al., 2010). During chronic psychological stress, SNS activation increased the proliferation and differentiation of HSCs into myeloid cells that was dependent on β3 adrenergic receptor expression on MSCs (Heidt et al., 2014). Thus, SNS activation during chronic nicotine consumption may increase HSC proliferation and myelopoiesis.

In this study, the effects of nicotine consumption on myelopoiesis and HSC proliferation were assessed in mice. To do this, leukocyte distribution and hematopoietic progenitor proliferation was assessed following two or three weeks of nicotine supplementation in drinking

water. Nicotine exposure primarily altered extramedullary hematopoiesis in the spleen and not the bone marrow.

Chapter 2: Methods

Animals

Female C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, ME). Mice were eight weeks old at start of experiments. All mice were housed in cages of three under a twelve-hour light/dark cycle. Food and water were administrated *ad libidum*. The University of Illinois Institutional Animal Care and Use Committee approved all procedures.

Nicotine supplementation

Mice received nicotine via water supplementation. Nicotine (Sigma Aldrich, catalog number: N3876) was diluted in water at a concentration of 100 µg/mL. Water at both control and treatment groups included 0.4% of sweetener (i.e. Sodium Saccharin from Louwolf). Water intake was monitored to secure that both groups were consuming it.

Guanethidine injections

Guanethidine (Santa Cruz Biotechnology, catalog number: sc-211570), a drug that prevents the release of catecholamines, was injected intraperitoneally (i.p.) daily during the time of the experiment (i.e. three weeks). Mice were scuffed by the nape of the neck and injected i.p. with 100 µL of solution at a dose of 50 mg/kg.

Nicotine and cotinine measurements

Serum nicotine and cotinine concentrations were determined in serum from an independent cohort of mice. Blood was collected immediately with EDTA-lined syringes by cardiac puncture following CO_2 asphyxiation. Whole blood was centrifuged at 13552 g (i.e. 11000 rpm) during five minutes using a refrigerated centrifuge (Eppendorf, Centrifuge 5417 C). Plasma was separated and

stored at -80°C. Samples were submitted for analysis to the Metabolomics Center at the University of Illinois to measure cotinine (i.e. nicotine predominant metabolite) and nicotine concentrations through LC/MS.

Isolation of Cells from BM, Spleen, and Blood

Tissues were collected immediately following CO_2 asphyxiation. Whole blood was collected with EDTA-lined syringes by cardiac puncture. Spleens were collected in ice-cold phosphate-buffered saline (PBS). Spleens were then scored with a surgical blade (Garvey, catalog number: 40475) and gently pressed through a 70 µm strainer (Falcon 352350) to obtain a single cell suspension. To collect bone marrow, the epiphyses of femurs were cut off and the marrow was flushed out with ice-cold Hanks' Balanced Salt Solution (HBSS, ThermoFisher, catalog number: 88284). Cells were washed with PBS, and then filtered through a 70 µm nylon cell strainer.

Flow Cytometry

Cells from the BM, spleen, and whole blood were phenotyped using flow cytometry. Fc receptors were blocked with anti-CD16/CD32 antibody (BioLegend). Cells were washed and then incubated with the appropriate antibodies (see key resources table) for 40 minutes at 4°C. Labeling of hematopoietic stem cells (HSCs) occurred prior to fixation with 4% PFA and permeabilized with a combination of 2% Triton-X and 20x DAPI. Cell suspensions were then analyzed on a 13 channel Attune NxT Acoustic Focusing Flow Cytometer. Data were compensated using single fluorescent-stained compensation beads (ThermoFisher) and analyzed using FlowJo software (Tree Star) and positive labeling for each antibody was determined based on isotype stained controls.

Statistical Analysis

Experiments with a single independent variable with more than two levels were analyzed using One-Way ANOVA (SPSS-27 IBM). Tukey's was used for *post hoc* comparisons. Experiments with only two experimental groups were analyzed by Student's T test, or if variances were not equal, then they were analyzed by non-parametric T-test. Results are depicted graphically in figures. All data are expressed as treatment means ± SEM.

Chapter 3: Results

To confirm that mice were properly ingesting and metabolizing nicotine, the serum concentrations of nicotine and its primary metabolite were measured. Nicotine supplementation significantly increased nicotine **(Fig. 1A)** and cotinine **(Fig. 1B)** in serum. Analyte concentrations were only detected in a subset of control treated samples, and this may have been due to technical variation of background levels.

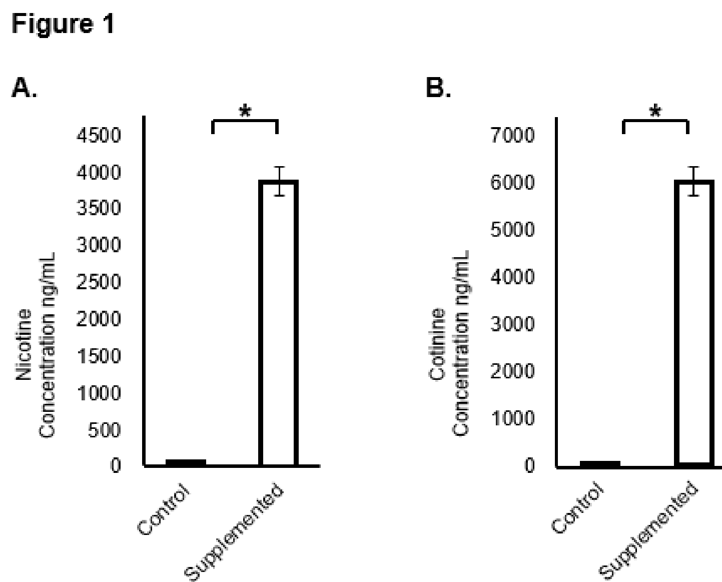

Figure 1: Nicotine supplementation increased nicotine and cotinine in serum. Circulating levels of (**A**) nicotine (P value = 0.00001) and **(B)** cotinine (P value = 0.0001) after ten days of nicotine supplementation via water consumption (N=12). Bars represent means ± SEMs. Means with asterisks are significantly different (* = $p < 0.05$).

To determine the effect of nicotine on myeloid cell production and distribution, mice were provided with 100 μg/mL nicotine or vehicle in drinking water for either two or three weeks. **Figure 2** shows the gating strategies used for quantification of Ly6C+ monocytes and granulocytes (**Fig. 2A**), CD4 and CD8 T cells (**Fig. 2B**), and B and NK cells (**Fig. 2C**). ANOVA did not reveal any main effects of nicotine duration on circulating leukocytes. However, *post hoc* analysis

revealed that three weeks of nicotine tended to decrease granulocytes in circulation (**Fig. 2D; p = 0.086**). Otherwise, nicotine had no effect on Ly6C^{hi} monocytes (**Fig. 2E**), CD4 T cells (**Fig. 2F**), CD8 T cells (**Fig. 2G**), B cells (**Fig. 2H**), or NK cells (**Fig. 2I**). Overall, nicotine supplementation did not significantly regulate leukocytes in circulation.

Figure 2

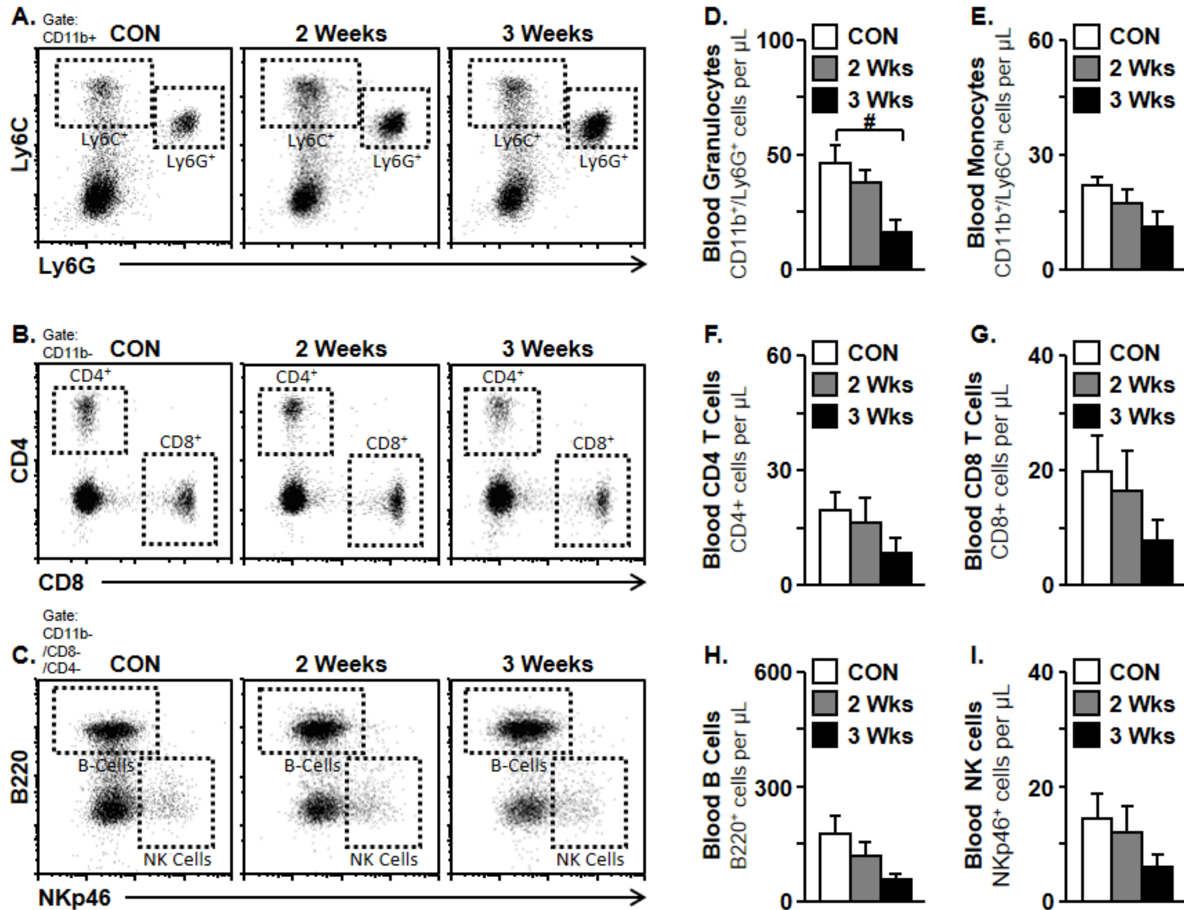

Figure 2: Nicotine supplementation did not alter circulating leukocytes. Flow cytometry analysis from blood samples after two and three weeks of nicotine supplementation. **(A)** Gating strategy for granulocytes and monocytes, **(B)** gating strategy for T cells, and **(C)** gating strategy for B Cells and NK Cells. Quantifications for **(D)** granulocytes [$F(2,6) = 1.86$, $p = 0.239$], **(E)** monocytes [$F(2,6) = 0.956$, $p = 0.436$], **(F)** CD4 T Cells [$F(2,6) = 0.635$, $p = 0.562$], **(G)** CD8 T Cells [$F(2,6) = 0.887$, $p = 0.460$], **(H)** B Cells [$F(2,6) = 0.297$, $p = 0.753$], and **(I)** NK Cells [$F(2,6) = 1.137$, $p = 0.381$]. Bars represent means ± SEMs. Means with asterisks are significantly different (* = $p < 0.05$). Means with number sign are a statistical trend (# = $0.1 > p \leq 0.05$).

Next, the effect of nicotine on splenic leukocyte counts was assessed. **Figure 3** shows the gating strategies used for quantification of splenic monocytes and granulocytes (**Fig. 3A**), CD4 and CD8 T cells (**Fig. 3B**), and B and NK cells (**Fig. 3C**). ANOVA did not reveal any significant main effects of nicotine duration on splenic leukocytes, but *post hoc* analysis revealed that three weeks of nicotine increased several leukocyte populations. Three weeks of nicotine increased granulocytes (**Fig. 3D**), monocytes **(Fig. 3E),** CD4 T cells (**Fig. 3F**), and CD8 T cells (**Fig. 3F**) in the spleen. Post hoc analysis did not reveal any effect of three weeks of nicotine on splenic B cells (**Fig., 3H**) or NK cells (**Fig. 3I**). Overall nicotine may have increased splenic monocytes, granulocytes, and T cells after three weeks of supplementation.

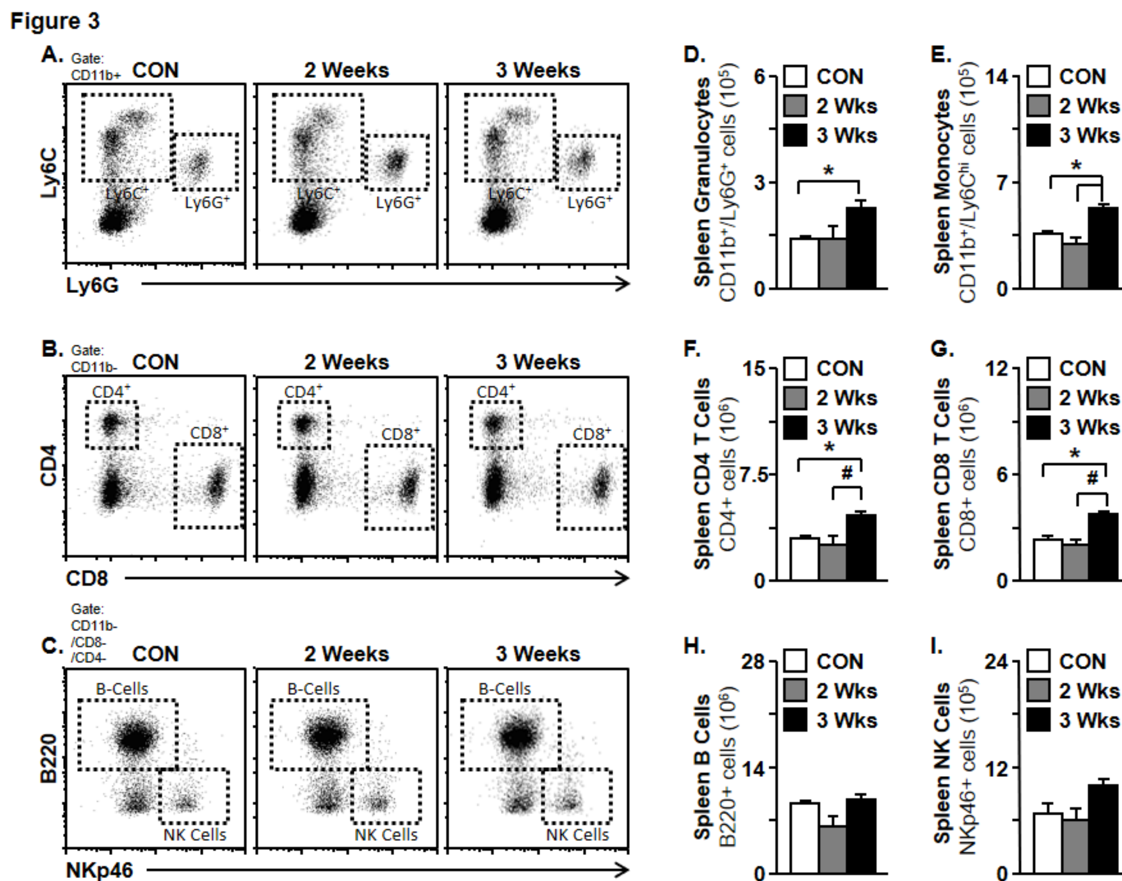

Figure 3: Nicotine supplementation may have increased splenic leukocytes. Flow cytometry analysis from spleen samples after two and three weeks of nicotine supplementation. **(A)** Gating

Figure 3 (cont.) strategy for granulocytes and monocytes, **(B)** gating strategy for T cells, and **(C)** gating strategy for B Cells and NK Cells. Quantifications for **(D)** granulocytes [F(2,6) = 0.375, p = 0.702], **(E)** monocytes [F(2,6) = 0.162, p = 0.854], **(F)** CD4 T Cells [F(2,6) =0.140, p = 0.872], **(G)** CD8 T Cells [F(2,6) = 0.191, p = 0.831], **(H)** B Cells [F(2,6) = 0.128, p = 0.883], and **(I)** NK Cells [F(2,6) = 0.382, p = 0.698]. Bars represent means ± SEMs. Means with asterisks are significantly different (* = $p < 0.05$). Means with number sign are a statistical trend (# = $0.1 > p \leq 0.05$).

Next, the effect of nicotine on bone marrow leukocyte counts was assessed. Figure 4 shows the gating strategies used for quantification of Ly6C monocytes and granulocytes (**Fig. 4A**), T cells (**Fig. 4B**), and B and NK cells (**Fig. 4C**). ANOVA did not reveal any significant main effects of nicotine duration on bone marrow leukocytes, but *post hoc* analysis revealed that three weeks of nicotine increased monocytes (**Fig. 4E**). Otherwise, nicotine did not affect bone marrow granulocytes (**Fig. 4D**), T cells (**Fig. 4F**), B cells (**Fig., 4G**), or NK cells (**Fig. 4H**).

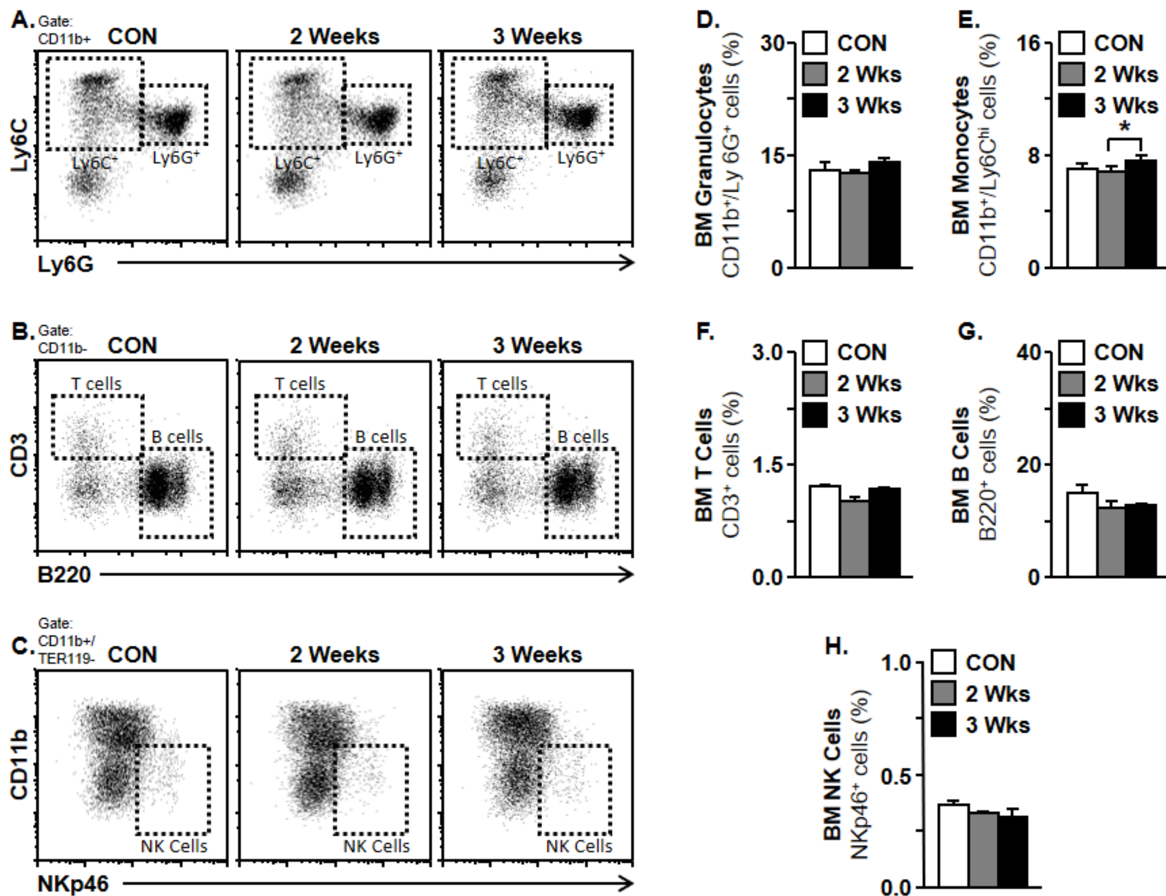

Figure 4 ***(cont.)*****: Nicotine supplementation did not affect bone marrow leukocytes.** Flow cytometry analysis from bone marrow samples after two and three weeks of nicotine supplementation. **(A)** Gating strategy for granulocytes and monocytes, **(B)** gating strategy for T cells, and **(C)** gating strategy for B Cells and NK Cells. Quantifications for **(D)** granulocytes [$F(2,6) = 1.263$, $p = 0.575$], **(E)** monocytes [$F(2,6) = 0.608$, $p = 0.575$], **(F)** T Cells [$F(2,6) = 0.255$, $p = 0.783$], **(G)** B Cells [$F(2,6) = 1.831$, $p = 0.240$], and **(H)** NK Cells [$F(2,6) = 0.193$, $p = 0.829$]. Bars represent means ± SEMs. Means with asterisks are significantly different (* = $p < 0.05$). Means with number sign are a statistical trend (# = $0.1 > p \leq 0.05$).

Next, the effect of nicotine on spleen and bone marrow cell cycle was assessed. Lineage negative cells, lacking expression of mature leukocyte markers, were gated on, and the expression of the hematopoietic progenitor markers, Sca1 and cKit, were determined by flow cytometry. Gating strategy for lineage negative populations expressing cKit (LK cells), Sca1 (LS cells), and both cKit and Sca1 (LSK cells) is shown in **Figure 5A**. Within these populations, DNA quantification using DAPI labeling was used to assess cell cycle. Non proliferating cells appear as the main peak in the DAPI histogram shown in **Figure 5B**, while proliferating cells in the S, G_2, and M phase exhibit increased DAPI fluorescence. ANOVA did not reveal any main effects of nicotine duration on proliferating progenitor populations in the spleen. However, *post hoc* analysis revealed that three weeks of nicotine increased proliferating LS and LK cells as a percentage of total splenocytes (**Fig. 5D** and **Fig. 5E,** respectively). Otherwise, nicotine did not effect proliferating LSK in the spleen (**Fig. 5C**) nor did it effect proliferating LSK cells (**Fig. 5F**), LS cells (**Fig. 5G**), or LK cells (**Fig. 5F**) in the bone marrow. Overall, nicotine may have increased proliferating hematopoietic progenitors in the spleen but not the bone marrow.

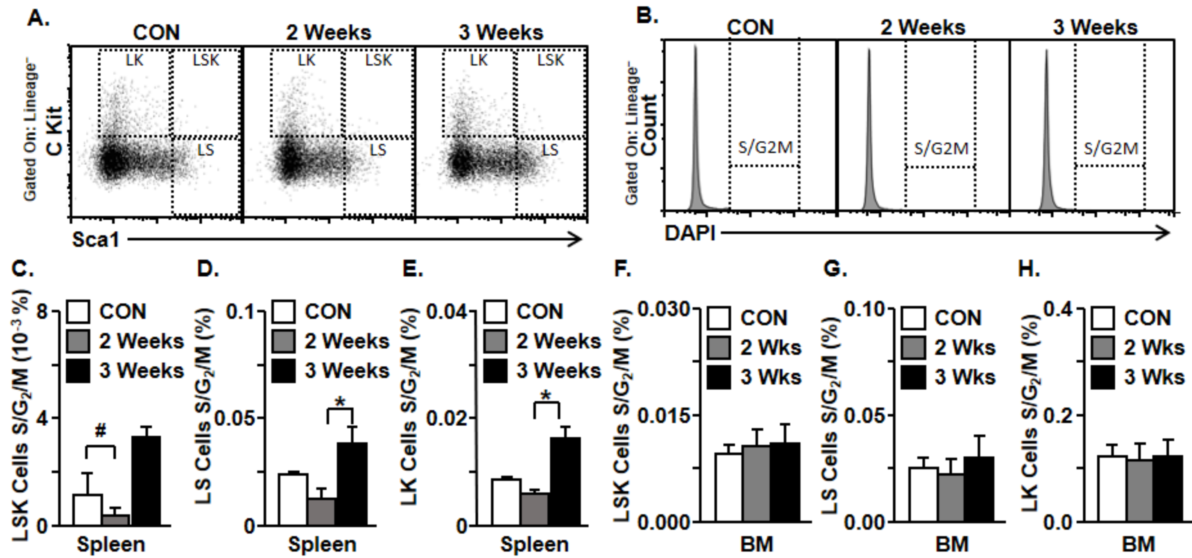

Figure 5: Nicotine supplementation may have increased proliferating hematopoietic progenitors in spleen but not bone marrow. Flow cytometry analysis from HSCs at bone marrow samples. **(A)** Gating strategy for cell cycle analysis, **(B)** graphs showing the gating strategy used to identify proliferating progenitors within the S, G_2, M phases of the cell cycle. Quantifications for **(C)** LSK Cells in the $S/G_2/M$ in spleen [$F(2,6) = 1.033$, $p = 0.412$], **(D)** LS Cells in the $S/G_2/M$ in spleen [$F(2,6) = 0.703$, $p = 0.532$], **(E)** LK Cells in the $S/G_2/M$ in spleen [$F(2,6) = 2.470$, $p = 0.165$], **(F)** LSK Cells in the $S/G_2/M$ in bone marrow [$F(2,6) = 0.090$, $p = 0.915$], **(G)** LS Cells in the $S/G_2/M$ in bone marrow [$F(2,6) = 1.896$, $p = 0.230$], and **(H)** LK Cells in the $S/G_2/M$ in bone marrow [$F(2,6) = 2.046$, $p = 0.210$]. Lineage included antibodies against CD11b, Ter119, CD71, IL7Ra, B220, CD3, CD4, CD8). Bars represent means ± SEMs. Means with asterisks are significantly different (* = $p < 0.05$). Means with number sign are a statistical trend (# = $0.1 > p \leq 0.05$).

To assess the role of SNS activity during nicotine supplementation, nicotine-treated mice were injected daily with the SNS-inhibitor, guanethidine. Guanethidine acts by displacing NE from its vesicles, preventing the release of NE. This approach is expected to remove the SNS-activating effects of nicotine. Mice were provided with 100 µg/mL nicotine or vehicle in drinking water for three weeks and received daily injections of either vehicle or guanethidine. **Figure 6** shows the gating strategies used for quantification of Ly6C monocytes and granulocytes (**Fig. 6A**), CD4 & CD8 T cells (**Fig. 6B**), and B and NK cells (**Fig. 6C**). Overall, nicotine and guanethidine combined had no effect on leukocyte counts in circulation. For instance, nicotine and guanethidine did not

affect circulating granulocytes (**Fig. 6D**), Ly6C^{hi} monocyte (**Fig. 6E**), CD4 T cells (Fig. 6F), CD4 T cells (**Fig. 6G**), B cells (**Fig., 6H**), or NK cells (**Fig. 6I**).

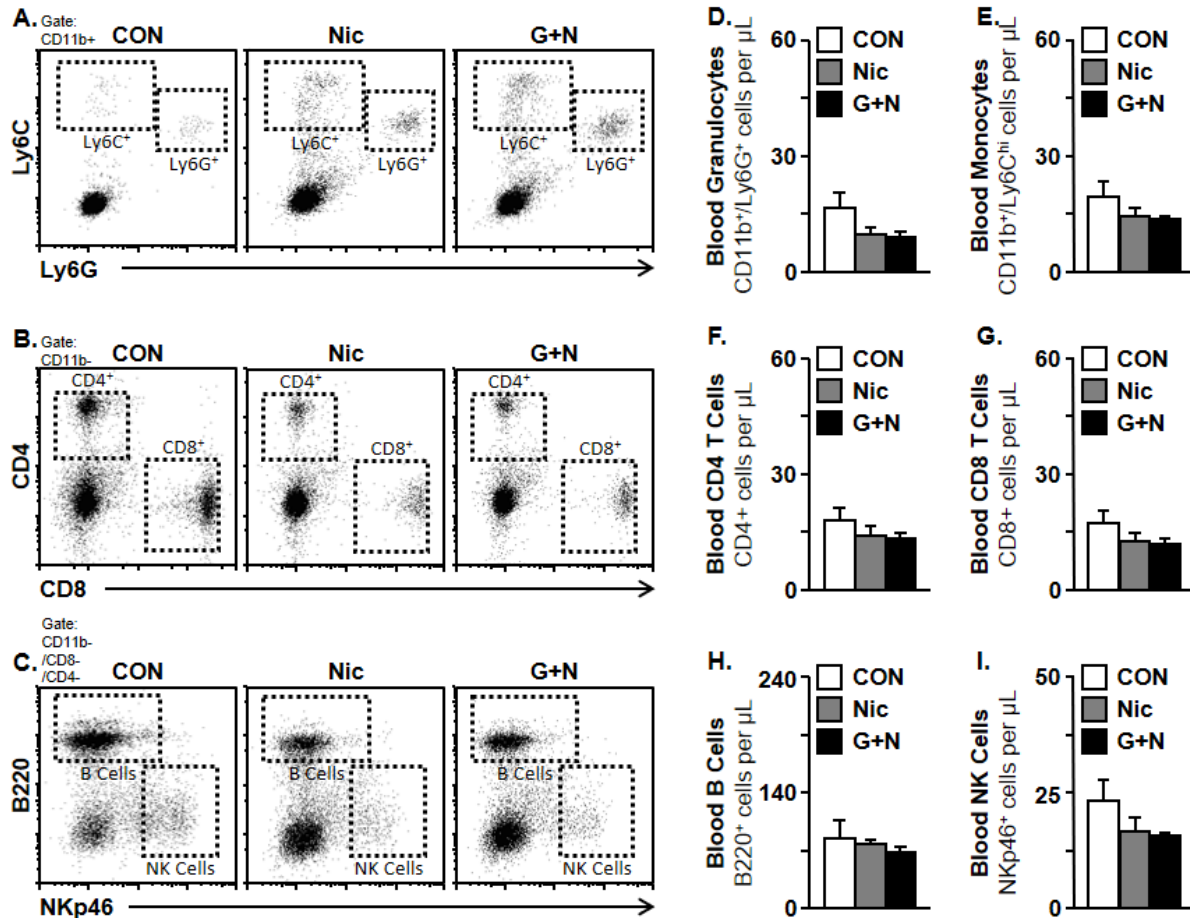

Figure 6: Nicotine and guanethidine supplementation did not affect circulating leukocytes. C57BL6 mice (N=9) were provided with drinking water supplemented with 100 ug/mL nicotine and received guanethidine or vehicle injections for three weeks, and distribution of circulating leukocytes was assessed by flow cytometry. Flow cytometry analysis from blood samples after supplementation. **(A)** Gating strategy for granulocytes and monocytes, **(B)** gating strategy for T cells, and **(C)** gating strategy for B Cells and NK Cells. Quantifications for **(D)** granulocytes [$F(2,6) = 3.720$, $p = 0.089$], **(E)** monocytes [$F(2,6) = 1.698$, $p = 0.260$], **(F)** CD4 T Cells [$F(2,6) = 0.870$, $p = 0.466$], **(G)** CD8 T Cells [$F(2,6) = 0.730$, $p = 0.520$], **(H)** B Cells [$F(2,6) = 1.864$, $p = 0.235$], and **(I)** NK Cells [$F(2,6) = 0.858$, $p = 0.470$]. Bars represent means ± SEMs. Means with asterisks are significantly different (* = $p < 0.05$).

Next, the effect of nicotine and guanethidine on bone marrow leukocyte counts was assessed. **Figure 7** shows the gating strategies used for quantification of Ly6C monocytes &

granulocytes (**Fig. 7A**), T cells (**Fig. 7B**), and B and NK cells (**Fig. 7C**). Overall, nicotine and guanethidine had no effect on leukocyte counts in spleen. For instance, nicotine and guanethidine did not affect granulocytes (**Fig. 7D**), Ly6C^{hi} monocyte (**Fig. 7E**), T cells (**Fig. 7F & G**), NK cells (**Fig. 7I**), or B-cells (**Fig. 7H**).

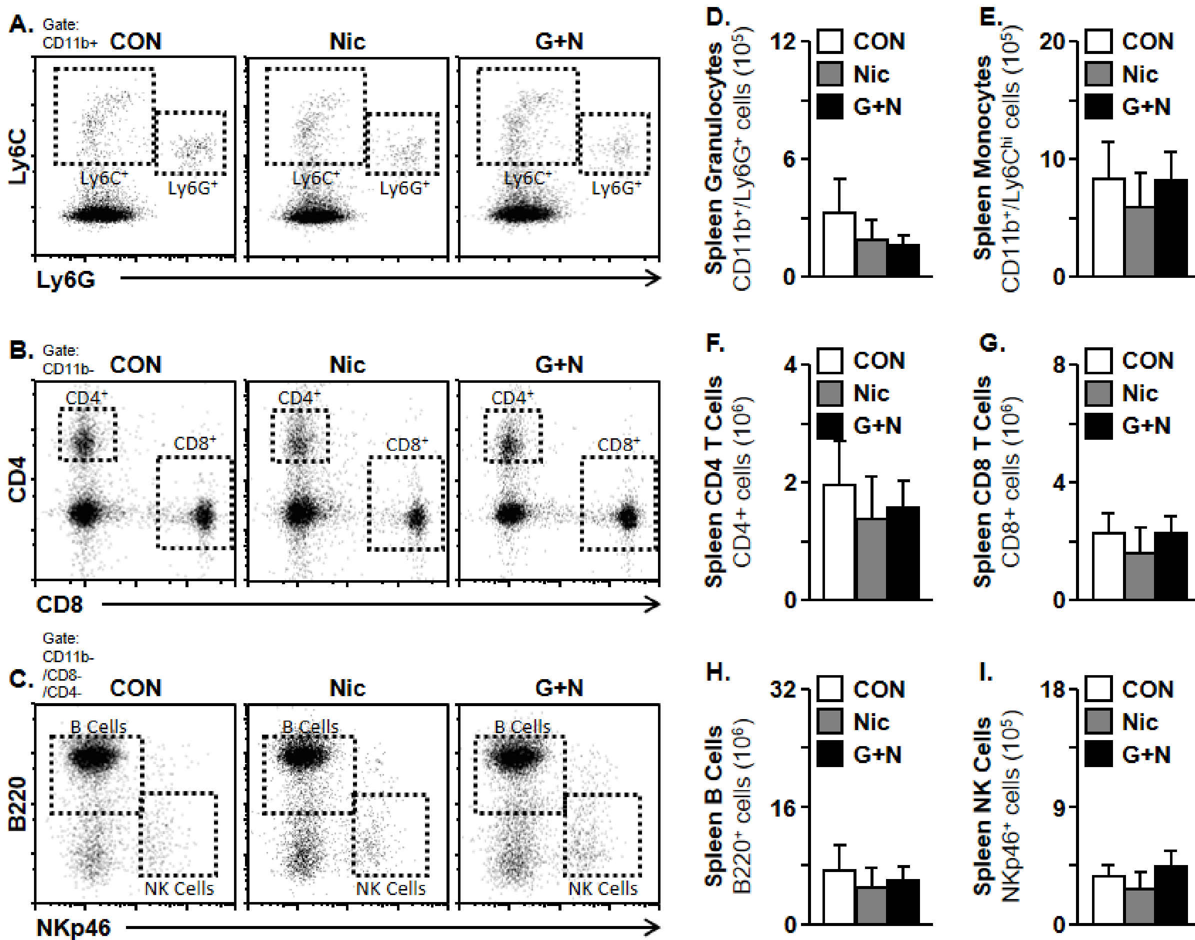

Figure 7: Nicotine and guanethidine supplementation did not affect splenic leukocytes. C57BL6 mice (N=9) were provided with drinking water supplemented with 100 µg/mL nicotine and received guanethidine or vehicle injections for three weeks, and distribution of leukocytes in spleen was assessed by flow cytometry. **(A)** Gating strategy for granulocytes and monocytes, **(B)** gating strategy for T cells, and **(C)** gating strategy for B Cells and NK Cells. Quantifications for **(D)** granulocytes [F(2,6) =, p =], **(E)** monocytes [F(2,6) =, p =], **(F)** CD4 T Cells [F(2,6) = 4.898, p = 0.55], **(G)** CD8 T Cells [F(2,6) = 9.598, p = 0.014], **(H)** B Cells [F(2,6) = 3.376, p = 0.104], and **(I)** NK Cells [F(2,6) = 2.401, p = 0.171]. Bars represent means ± SEMs. Means with asterisks are significantly different (* = $p < 0.05$).

Next, the effect of nicotine and guanethidine on bone marrow leukocyte counts was assessed. **Figure 8** shows the gating strategies used for quantification of Ly6C monocytes and granulocytes (**Fig. 8A**), T cells (**Fig. 8B**), and B and NK cells (**Fig. 8C**). Overall, nicotine and guanethidine combined had no effect on leukocyte counts in bone marrow. For instance, nicotine did not affect granulocytes (**Fig. 8D**), monocytes (**Fig. 8E**), T cells (**Fig. 8F**), NK cells (**Fig. 8G**) nor B-cells (**Fig. 8H).**

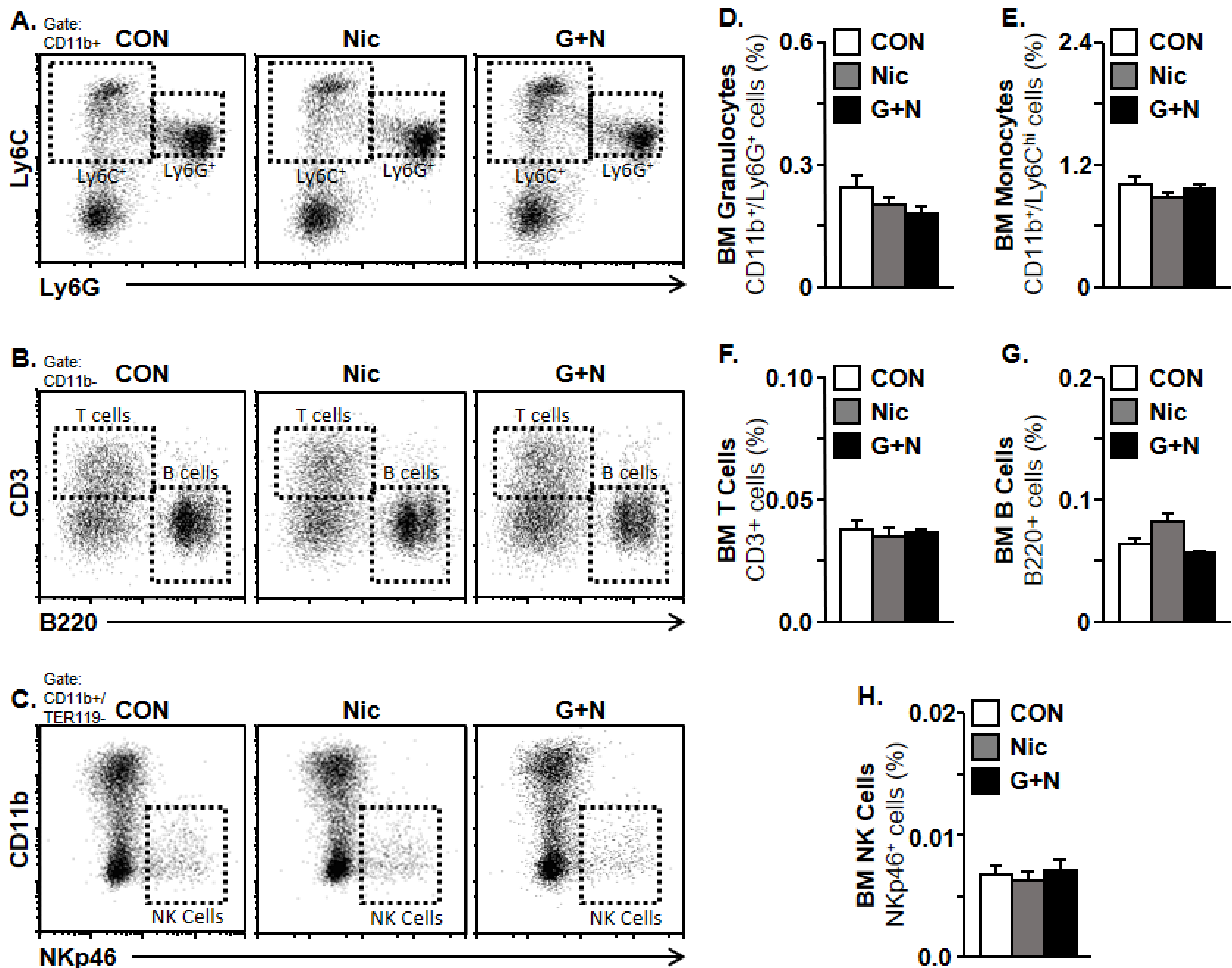

Figure 8: Nicotine and guanethidine supplementation did not affect bone marrow leukocytes. C57BL6 mice (N=9) were provided with drinking water supplemented with 100 ug/mL nicotine and received guanethidine or vehicle injections for three weeks, and distribution of leukocytes in bone marrow was assessed by flow cytometry. **(A)** Gating strategy for granulocytes and

Figure 8 (cont.) monocytes, **(B)** gating strategy for T cells, and **(C)** gating strategy for B Cells and NK Cells. Quantifications for **(D)** granulocytes [$F(2,6) = 0.363$, $p = 0.716$], **(E)** monocytes [$F(2,6) = 0.458$, $p = 0.662$], **(F)** T Cells [$F(2,6) = 1.837$, $p = 0.272$], **(G)** B Cells [$F(2,6) = 1.005$, $p = 0.443$], and **(H)** NK Cells [$F(2,6) = 0.462$, $p = 0.660$]. Bars represent means ± SEMs. Means with asterisks are significantly different (* = $p < 0.05$).

Next, the effect of nicotine and guanethidine combined on spleen and bone marrow cells' cycles was assessed. Gating strategies for lineage negative populations expressing cKit (LK), Sca1 (LS), and both cKit and Sca1 (LSK) is shown in **Figure 9A**. Quantification of cellular DNA by DAPI labeling was used to determine cell cycles of progenitor populations **(Fig. 9B)**. Overall, neither nicotine nor guanethidine affected proliferating hematopoietic progenitors in either spleen or bone marrow. For instance, there was no effect on proliferating splenic LSK cells (**Fig. 9C**), LS cells (**Fig. 9D**), or LK cells (**Fig. 9E**). Similarly, there was no effect on proliferating bone marrow LSK cells (**Fig. 9F**), LS cells (**Fig. 9G**), or LK cells (**Fig. 9H**).

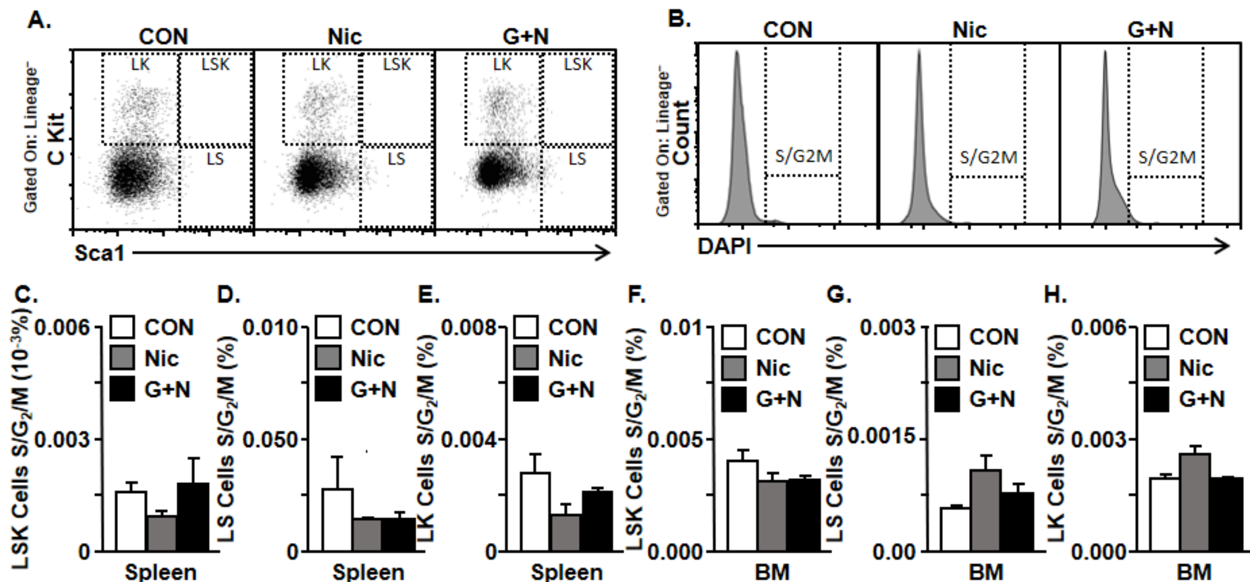

Figure 9: Nicotine and guanethidine supplementation did not affect proliferating hematopoietic progenitors. C57BL6 mice (N=9) were provided with drinking water supplemented with 100 ug/mL nicotine and received guanethidine or vehicle injections for three weeks, and distribution of HSC in bone marrow was assessed by flow cytometry. **(A)** Gating strategy for cell cycle analysis, **(B)** graphs showing the gating strategy used to identify proliferating

Figure 9 (cont.) progenitors within the S, G_2, M phases of the cell cycle. Quantifications for **(C)** LSK Cells in the S/G_2/M in spleen [$F(2,5) = 5.439$, $p = 0.056$], **(D)** LS Cells in the S/G_2/M in spleen [$F(2,5) = 5.956$, $p = 0.048$], **(E)** LK Cells in the S/G_2/M in spleen [$F(2,5) = 6.219$, $p = 0.044$], **(F)** LSK Cells in the S/G_2/M in bone marrow [$F(2,6) = 0.082$, $p = 0.922$], **(G)** LS Cells in the S/G_2/M in bone marrow [$F(2,6) = 0.070$, $p = 0.933$], and **(H)** LK Cells in the S/G_2/M in bone marrow [$F(2,6) = 0.004$, $p = 0.996$]. Lineage included antibodies against CD11b, Ter119, CD71, IL7Ra, B220, CD3, CD4, CD8). Bars represent means ± SEMs. Means with asterisks are significantly different (* = $p < 0.05$).

Chapter 4: Discussion

Overall, nicotine supplementation minimally influenced leukocyte production and distribution. Generally, the effects of nicotine supplementation on leukocytes was characteristically inconsistent with previous reports of increased myelopoiesis caused by nicotine and smoking. Several confounders may account for the lack of effect of nicotine supplementation on myelopoiesis. For instance, the specific dose of nicotine may have contributed to tolerance and abrogated the effects of chronic nicotine supplementation on SNS activation. Alternatively, nicotine supplementation over longer periods of time may increasingly influence and restructure hematopoiesis. Similarly, species differences between mice and humans, in terms of lifespan and metabolism, may also account for discrepancies between results here and results from human studies. It is also possible that combustion related particulates and not nicotine may account for the association between smoking and myelopoiesis. Despite these possibilities, serum concentrations of nicotine and cotinine confirmed mice were consuming and metabolizing the provided nicotine. Thus, under these conditions nicotine did not increase myelopoiesis in such a way that resulted in systemically increased myeloid cells. While nicotine did not systemically influence myelopoiesis, splenic hematopoiesis and myeloid cells were increased by nicotine. Over extended periods of time nicotine-induced splenic hematopoiesis may influence health outcomes. Collectively, two and three weeks of nicotine supplementation did not systemically increase myelopoiesis mice.

Development of nicotine tolerance may have abrogated the effects of nicotine on SNS activation and myelopoiesis in the current study. Nicotine tolerance initiates within rapidly upon exposure (Collins et al., 1988). Despite this, another study in mice similarly reported that nicotine supplementation increased extramedullary hematopoiesis in the spleen after three weeks (Pandit

et al., 2006). Therefore, despite the possibility of tolerance, nicotine appears to reproducibly increase splenic hematopoiesis in mice. Regarding to this point it is important to mention that humans and mice often exhibit differential degrees of drug metabolism and receptor tolerance. Similarly, human smokers often consume nicotine for decades, and these relative durations cannot be readily modeled in rodents with dramatically shorter lifespans. For these reasons, it remains possible that nicotine mediates the myelopoietic effects of smoking in humans despite the absence of effects in mice in the current study.

Notably, nicotine increased hematopoietic proliferation and leukocyte numbers in the spleen, but this was not reflected by increased leukocytes in the blood or bone marrow. It is possible that longer durations of nicotine exposure may have increased circulating leukocytes that derived from the spleen. In order to observe an increase of leucocytes at circulation it may be appropriate to increase nicotine supplementation for longer periods of time. Such experiments would more faithfully mimic the relatively high proportions of human lifespans that many individuals continuously smoke tobacco (e.g., decades).

Similar to the effects of nicotine, we reported that SNS activation during social stress in mice also increased splenic hematopoiesis (McKim et al., 2018). This is consistent with notion that nicotine-induced splenic hematopoiesis may also be SNS dependent. Unfortunately, the experiment reported here that involved guanethidine administration to nicotine treated mice did not reproduce the observation of nicotine-induced splenic hematopoiesis. For this reason, the current study failed to assess the role of SNS activity. The notable lack of splenic hematopoiesis in vehicle injected nicotine treated mice may have been caused by the stress associated with daily vehicle injections. Future studies may address this by including a nicotine group that does not receive daily vehicle injections.

Despite numerous caveats discussed here, elevated nicotine and cotinine in serum confirmed that experimental mice were successfully ingesting and metabolizing the provided nicotine. This is important because it excludes the possibility that experimenter or dosing error was responsible for the lack of effects of nicotine supplementation. The nicotine dose that was used here (i.e. 100 μg/mL) is relatively high compared to the mass per body weight consumption of nicotine in humans. However, the use of relatively high nicotine dose excludes the possibility that sub-physiological levels were achieved. Another study in mice tested oral nicotine consumption in a range from 10 to 200 ug/mL, and they reported that this dose was well tolerated and non-toxic (Klein et al., 2004). However, a limitation of our experiment relies in the fact that the time that the mice received nicotine supplementation may not be representative of chronic nicotine consumption in humans. Despite this, another study similarly showed that three weeks of nicotine supplementation also increased extramedullary hematopoiesis in the spleen (Pandit et al., 2006). Thus, despite some notable caveats, it is evident that data reported faithfully represents the effects of nicotine within current experimental parameters.

Interestingly there is some evidence that splenic hematopoiesis may contribute to atherosclerosis. For instance, splenic hematopoiesis correlated with atherosclerotic lesion inflammation and predicted future cardiovascular events (Tawakol et al., 2017). This is notable because splenic hematopoiesis may increase the supply of myeloid cells to inflamed arteries in the context of concurrent atherosclerosis. For this reason, further preclinical investigation into the role of nicotine-induced splenic hematopoiesis in atherosclerosis may be warranted.

Despite the notable lack of systemically increased myeloid cells in nicotine-treated mice, our results are consistent with previous literature regarding nicotinic activation of splenic hematopoiesis. Caveats related to duration, dose, and tolerance may be addressed by future studies.

However, the observation that nicotine reproducibly increased splenic hematopoiesis may have implications for the association between smoking and cardiovascular disease.

References:

Agarwala, S., & Tamplin, O. J. (2018). Neural Crossroads in the Hematopoietic Stem Cell Niche. *Trends in Cell Biology*, *28*(12), 987–998. https://doi.org/10.1016/J.TCB.2018.05.003

Aguila, H. L. (2006). Regulation of hematopoietic niches by sympathetic innervation. *BioEssays*, *28*(7), 687–691. https://doi.org/10.1002/BIES.20427

Andreoli, C., Bassi, A., Gregg, E. O., Nunziata, A., Puntoni, R., & Corsini, E. (2015). Effects of cigarette smoking on circulating leukocytes and plasma cytokines in monozygotic twins. *Clinical Chemistry and Laboratory Medicine*, *53*(1), 57–64. https://doi.org/10.1515/cclm-2013-0290

Benowitz, N. L., Gourlay, S. G., & Francisco, S. (1997). Cardiovascular Toxicity of Nicotine: Implications for Nicotine Replacement Therapy. *J Am Coll Cardiol*, *29*, 1422–1453. https://doi.org/10.1016/S0735-1097

Centner, A. M., Bhide, P. G., & Salazar, G. (2020). Nicotine in Senescence and Atherosclerosis. *Cells*, *9*(4). https://doi.org/10.3390/CELLS9041035

Collins, A., Romm, E., & Wehner, J. (1988). Nicotine tolerance: an analysis of the time course of its development and loss in the rat. *Psychopharmacology*, *96*(1), 7–14. https://doi.org/10.1007/BF02431526

Flynn, M. C., Kraakman, M. J., Tikellis, C., Lee, M. K. S., Hanssen, N. M. J., Kammoun, H. L., Pickering, R. J., Dragoljevic, D., Al-Sharea, A., Barrett, T. J., Hortle, F., Byrne, F. L., Olzomer, E., McCarthy, D. A., Schalkwijk, C. G., Forbes, J. M., Hoehn, K., Makowski, L., Lancaster, G. I., … Murphy, A. J. (2020). Transient Intermittent Hyperglycemia Accelerates Atherosclerosis by Promoting Myelopoiesis. *Circulation Research*, *127*(7), 877–892. https://doi.org/10.1161/CIRCRESAHA.120.316653

Haass, M., & Kubler, W. (1996). Nicotine and Sympathetic Neurotransmission. In *Cardiovascular Drugs and Therapy* (Vol. 10).

Heidt, T., Sager, H. B., Courties, G., Dutta, P., Iwamoto, Y., Zaltsman, A., von zur Muhlen, C., Bode, C., Fricchione, G. L., Denninger, J., Lin, C. P., Vinegoni, C., Libby, P., Swirski, F. K., Weissleder, R., & Nahrendorf, M. (2014). Chronic variable stress activates hematopoietic stem cells. *Nature Medicine*. https://doi.org/10.1038/nm.3589

Jensen, E. J., Pedersen, B., Frederiksen, R., & Dahl, R. (1998). Prospective study on the effect of smoking and nicotine substitution on leucocyte blood counts and relation between blood leucocytes and lung function. *Thorax*, *53*(9), 784–789. https://doi.org/10.1136/thx.53.9.784

Katayama, Y., Battista, M., Kao, W. M., Hidalgo, A., Peired, A. J., Thomas, S. A., & Frenette, P. S. (2006). Signals from the sympathetic nervous system regulate hematopoietic stem cell egress from bone marrow. *Cell*, *124*(2), 407–421. https://doi.org/10.1016/j.cell.2005.10.041

Klein, L. C., Stine, M. M., Vandenbergh, D. J., Whetzel, C. A., & Kamens, H. M. (2004). Sex differences in voluntary oral nicotine consumption by adolescent mice: a dose-response experiment. *Pharmacology Biochemistry and Behavior*, *78*(1), 13–25. https://doi.org/10.1016/J.PBB.2004.01.005

Lee, J.-E., & Cooke, J. P. (2011). The role of nicotine in the pathogenesis of atherosclerosis. *Atherosclerosis*, *215*(2), 281. https://doi.org/10.1016/J.ATHEROSCLEROSIS.2011.01.003

McKim, D. B., Yin, W., Wang, Y., Cole, S. W., Godbout, J. P., & Sheridan, J. F. (2018). Social Stress Mobilizes Hematopoietic Stem Cells to Establish Persistent Splenic Myelopoiesis. *Cell Reports*, *25*(9), 2552-2562.e3. https://doi.org/10.1016/j.celrep.2018.10.102

Méndez-Ferrer, S., Battista, M., & Frenette, P. S. (2010). Cooperation of β2- and β3-adrenergic receptors in hematopoietic progenitor cell mobilization. *Annals of the New York Academy of Sciences*, *1192*, 139–144. https://doi.org/10.1111/j.1749-6632.2010.05390.x

Méndez-Ferrer, S., Lucas, D., Battista, M., & Frenette, P. S. (2008). Haematopoietic stem cell release is regulated by circadian oscillations. *Nature*, *452*(7186), 442–447. https://doi.org/10.1038/nature06685

Méndez-Ferrer, S., Michurina, T. V., Ferraro, F., Mazloom, A. R., MacArthur, B. D., Lira, S. A., Scadden, D. T., Ma'ayan, A., Enikolopov, G. N., & Frenette, P. S. (2010). Mesenchymal and haematopoietic stem cells form a unique bone marrow niche. *Nature*, *466*(7308), 829–834. https://doi.org/10.1038/nature09262

Nagareddy, P. R., Murphy, A. J., Stirzaker, R. A., Hu, Y., Yu, S., Miller, R. G., Ramkhelawon, B., Distel, E., Westerterp, M., Huang, L. S., Schmidt, A. M., Orchard, T. J., Fisher, E. A., Tall, A. R., & Goldberg, I. J. (2013). Hyperglycemia promotes myelopoiesis and impairs the resolution of atherosclerosis. *Cell Metabolism*, *17*(5), 695–708. https://doi.org/10.1016/j.cmet.2013.04.001

Pandit, T. S., Sikora, L., Muralidhar, G., Rao, S. P., & Sriramarao, P. (2006). Sustained Exposure to Nicotine Leads to Extramedullary Hematopoiesis in the Spleen. *Stem Cells*, *24*(11), 2373–2381. https://doi.org/10.1634/stemcells.2005-0447

Swirski, F. K., Libby, P., Aikawa, E., Alcaide, P., Luscinskas, F. W., Weissleder, R., & Pittet, M. J. (2007). Ly-6Chi monocytes dominate hypercholesterolemia-associated monocytosis and give rise to macrophages in atheromata. *Journal of Clinical Investigation*, *117*(1), 195–205. https://doi.org/10.1172/JCI29950

Tawakol, A., Ishai, A., Takx, R. A., Figueroa, A. L., Ali, A., Kaiser, Y., Truong, Q. A., Solomon, C. J., Calcagno, C., Mani, V., Tang, C. Y., Mulder, W. J., Murrough, J. W., Hoffmann, U., Nahrendorf, M., Shin, L. M., Fayad, Z. A., & Pitman, R. K. (2017). Relation between resting amygdalar activity and cardiovascular events: a longitudinal and cohort study. *The Lancet*, *389*(10071), 834–845. https://doi.org/10.1016/S0140-6736(16)31714-7

Yvan-Charvet, L., Pagler, T., Gautier, E. L., Avagyan, S., Siry, R. L., Han, S., Welch, C. L., Wang, N., Randolph, G. J., Snoeck, H. W., & Tall, A. R. (2010). ATP-binding cassette transporters and HDL suppress hematopoietic stem cell proliferation. *Science*, *328*(5986), 1689–1693. https://doi.org/10.1126/science.1189731

Appendix: Key Resources Table		
Reagent or Sources	**Source**	**Catalog number**
Antibodies		
Rat Anti-Human/Anti-Mouse B220 (CD45R) Super Bright 600 (Clone: RA3-6B2)	eBioscience	63-0452-82
Rat Anti-Mouse Ly-6G Brilliant Violet 650 (Clone: 1A8)	Biolegend	127641
Rat Anti-Mouse CD11b Super Bright 702 (Clone: M1/70)	eBioscience	67-0112-82
Rat Anti-Mouse CD4 Super Bright 780 (Clone: GK1.5)	eBioscience	78-0041-82
Rat Anti-Mouse CD3 Brilliant Violet 785 (Clone: 17A2)	Biolegend	100232
Rat Anti-Mouse TER-119 PerCP-eFluor 710 (Clone: TER-119)	eBioscience	46-5921-82
Rat Anti-Mouse Ly-6C PE (Clone: HK1.4)	Biolegend	128008
Rat Anti-Mouse CD8a PE-Cy7 (Clone: 53-6.7)	eBioscience	25-0081-82
Rat Anti-Mouse CD16/32 PE-Cy7 (Clone: 93)	eBioscience	25-0161-82
Rat Anti-Mouse CD335 (NKp46) APC (Clone: 29A1.4)	eBioscience	17-3351-82
Rat Anti-Mouse CD150 APC (Clone: mShad150)	eBioscience	17-1502-80
Rat Anti-Mouse CD71 (Transferrin Receptor) Alexa Fluor 700 (Clone: R17217)	eBioscience	56-0711-80
PE/Dazzle 594 Streptavidin	Biolegend	405248
Rat Anti-Mouse CD117 (c-Kit) Super Bright 780 (Clone: 2B8)	eBioscience	78-1171-82
Rat Anti-Mouse Ly-6A/E (Sca-1) FITC (Clone: 30-F11)	eBioscience	11-5981-81
Armenian Hamster Anti-Mouse CD48 PerCP-eFluor 710 (Clone: HM48-1)	eBioscience	46-0481-82
Rat Anti- Mouse CD34 PE (Clone: SA376A4)	BioLegend	152204
Rat Anti-Mouse Ly-6C APC eFluor 780 (Clone: HK1.4)	eBioscience	47-5932-82
Rat Anti-Human/Anti-Mouse B220 (CD45R) Biotin (Clone: RA3-6B2)	BioLegend	103204
Rat Anti-Mouse CD4 Biotin (Clone: GK1.5)	BioLegend	100404
Rat Anti-Mouse CD8a (Clone: 53-6.7)	Biolegend	100704
Armenian Hamster Anti-Mouse CD3e Biotin (Clone: 145-2C11)	Biolegend	100304
Rat Anti-Mouse TER-119 Biotin (Clone: TER-119)	BioLegend	116204
Rat Anti-Mouse CD127 (IL-7Ra) Biotin (Clone: A7R34)	eBioscience	13-1271-85

Rat Anti-Mouse CD41a Biotin (Clone: MWReg30)	eBioscience	13-0411-85
TruStain FcX (anti-mouse CD16/32) Antibody (Clone: 93)	BioLegend	101320
Chemicals and Reagents		
DAPI	Sigma-Aldrich	D9542
UltraComp eBeads Compensation Beads	ThermoFisher	01-2222-42
eBioscience Super Bright Complete Staining Buffer	ThermoFisher	SB-4401-75
-/- Dulbecco's Phosphate Buffered Saline	ThermoFisher/Gibco	21600-04
UltraPure 0.5 M EDTA, pH 8.0	ThermoFisher	15575-038
ACK Lysing Buffer	ThermoFisher/Gibco	A10492-01
(-)-Nicotine	Sigma-Aldrich	N3876
Guanethinedine	Santa Cruz Biotechnology	sc-211570
Experimental Models: Organisms/Strains		
C57BL/6	Jackson Laboratory (Bar Harbor, ME)	000664
Software		
FlowJo Software	Treestar	RRID: SCR_008520
Equipment		
Attune NxT, Acoustic Focusing Cytometer	ThermoFisher	AFC2
70-micron cell strainer	Dot Scientific	667483
23G Needles	BD Biosciences	14-821-13F