

SUPPLEMENTAL METHODS

Gene Expression Analysis

RNA preparation for microarray. RNA was isolated from sorted pro- and pre- B-cell progenitors using the RNeasy Plus Micro Kit (Qiagen; Cat. No. 74034). ST-cDNA was generated using the Ovation RNA Amplification System V2 (NuGen), and ST-cDNA was amplified and prepared for hybridization using the WT-Ovation Exon Module (NuGen) both per the manufacturer's protocols. Fragmentation and biotinylation of the samples were achieved using the FL-Ovation cDNA Biotin Module V2 (NuGen) per the manufacturer's instructions. Prior to gene expression profiling, the quality of all samples were confirmed using the Agilent 2100 Bioanalyzer (Agilent Technologies, Inc.).

Gene expression profiling. RNA levels were profiled using Affymetrix Mouse Gene 1.0 ST Array. Probe sets level data were preprocessed and normalized using Robust Multiarray Average (RMA) method (1), available in the BioConductor package. Probe sets representing the same gene were collapsed based on maximum values.

Differentially expressed genes analysis. We used the signal-to-noise ratio (SNR; ref4) to identify differential expressed genes in each comparison. Let $[\mu_1(g), \mu_2(g)]$ and $[\sigma_1(g), \sigma_2(g)]$ denote the means and SDs of the log of the expression levels of gene g . The SNR used is based on the null hypothesis that mean of expression level in group 1 is the same as the one in the group 2 (

$H_0 : \mu_1(g) = \mu_2(g), H_1 : \mu_1(g) \neq \mu_2(g)$). For each gene g , SNR is obtained by

$SNR_g = \frac{\mu_1(g) - \mu_2(g)}{\sigma_1(g) + \sigma_2(g)}$, where 1 is group 1 and 2 is group 2. The SNR_g reflects the difference between

the groups relative to the SD within the groups. Large values of $|SNR_g|$ indicate a strong correlation

between the gene expression and the group distinction, while the sign of SNR_g being positive or negative corresponds to g being more highly expressed in group 1 or group 2.

Gene set enrichment analysis. Analysis of microarray data was performed using the gene set enrichment analysis (GSEA) software (2) version 2.0.6 obtained from Broad Institute

(<http://www.broadinstitute.org/>). The gene sets database was compiled from the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (3). The KEGG gene sets database contains 210 mouse pathways that include metabolism, genetic information processing, environmental information processing, cellular processes, and human diseases. One hundred eight-eight gene sets passed the gene set size filter criteria (min, 10; max, 500). P values for the gene sets were computed by permuting the gene sets 1,000 times in this study.

Ingenuity analysis.

The microarray data were analyzed using the Ingenuity Pathway Analysis (Qiagen) in order to identify pathways that significantly change in B-cell progenitors with age.

qRT-PCR Analysis

qRT-PCR analysis of B-progenitor populations was performed using previously established protocols (4). Briefly, RNA was isolated from either B220-purified or sorted B-cell progenitors using the RNeasy Plus Micro Kit (Qiagen; Cat. No. 74034), followed by cDNA generation using the First Strand Synthesis Kit (Roche; Ref. No. 04 896 866 001). PrimeTime qPCR Assay reagents (IDT) were used for the murine genes HPRT (left: 5'-TCC TCC TCA GAC CGC TTT T-3' / right: CCT GGT TCA TCA TCG CTA ATC-3'; Prb69), GMPS (left: 5'-GTG CAA CGG AGA TTC CAA G-3' / right: 5'-TCG TAA TGG TGA GAA CCA TCC-3'; Prb25), cMYC (left: 5'-GCC TAG AAT TGG CAG AAA TGA-3' / right: 5'-AAC TGA GAA GAA TCC TAT TCA GCA C-3'; Prb63) EBF (left: 5'-CAG CTG CCA ACT CAC CCT AT-3' / right: 5'-TGG TAG ATG AGG CCA TGG TG-3'; Prb42), and

Pax5 (left: 5'-ACG CTG ACA GGG ATG GTG-3'/ right: GGG GAA CCT CCA AGA ATC AT-3'; Prb83). Applied Biosystems TaqMan Gene Expression Assays were used to detect murine GAPDH (Cat. No. Mm99999915_g1), Muc5b (Cat. No. Mm00466391_m1), interferon-inducible protein-27 (Cat. No. Mm00835449_g1), interferon- ζ (Cat. No. Mm02525738_g1), and TNF- α (Cat. No. Mm00443260_g1). qPCR was performed using a LightCycler 480 (Roche).

Mass Spectrometry Analysis

Sample preparation:

An average of 1.5×10^6 cells was lysed with 6 mL ice-cold PCA (8%) as described previously (5). The samples were centrifuged, the liquid phase removed and neutralized to a pH of 7.0-7.3 using KOH. To separate from perchlorate salts, the neutralized samples were centrifuged again, and the supernatant lyophilized overnight.

LC/LC-MS/MS: We determined high-energy phosphate metabolite concentrations in using a previously described modified assay (5). An Agilent series 1100 HPLC (Agilent Technologies, Palo Alto, CA) coupled to an API4000 triple quadrupole mass spectrometer (AB Sciex, Concord, ON) equipped with an electrospray ionization (ESI) source was employed for quantitation of nucleotide mono-, di-, and triphosphates. Lyophilisates (vide supra) were re-dissolved in 0.5 ml water containing 6-aminoethyl-ADP (as internal standard). 50 μ l of extract were loaded onto the C18 cartridge desalting column (50 \times 4.6 mm Zorbax C18, 5 μ m particle size, Agilent Technologies) and were washed with 1 mL/min of 97% 4 mmol/L dibutylammonium formate (DBAF) buffer and 3% methanol for 0.8 min. Thereafter, the switching valve was activated and the analytes were back-flushed from the desalting column onto the analytical column (250 \times 4.6 mm Phenomenex Synergy Hydro C18, 3 μ m particle size, Phenomenex, Torrance, CA, USA) with 0.5 mL/min. The nucleotide peak separation was performed using the 2 mmol/L

DBAF buffer (as solvent A) and 5% methanol (as solvent B) with the following gradient: from 95% to 88% solvent A within the first 3 min, from 88% to 65% solvent A from 3-15 minutes, and to 20% solvent A for the subsequent 2 min. The following 4 min were used to re-equilibrate the analytical column to the starting conditions. The flow rate was 0.7 mL/min, and the column temperature was maintained at 35 °C. Nucleotides were quantified by calculating the nucleotide area-under-the-peak/internal standard under-the-peak ratios and comparing with those of included calibration curves.

Untargeted Quantitative ^1H -Nuclear Magnetic Resonance Metabolomics Analysis

Ultra-Small Volume Quantitative NMR Spectroscopy: Isolated B-cell progenitors from pooled animals (3 independent experiments; 5 pooled animals/ group; sample size: 8-12 mg) were extracted with 0.2 mL of ice cold methanol/chloroform mixture as previously described (6). The water-soluble fractions were lyophilized overnight and re-dissolved in 30 μL D_2O with deuterated trimethyl-silyl-propionic acid (d -TMSP, as an external standard). The extracts were transferred into Bruker 1-mm glass capillaries and inserted into the magnet using a 1-mm NMR spinner.

All NMR experiments were performed on a Bruker 500 MHz spectrometer (operating frequency 500.15 MHz) using a Bruker ultra-small volume 1-mm high-resolution inverse TXI ($^1\text{H}/^{13}\text{C}/^{31}\text{P}$) Z-gradient microprobe (Bruker BioSpin, Billerica, MA). One dimensional ^1H -NMR spectra were obtained from each sample, with a standard water pre-saturation pulse program “zgpr” (6). The pulse delay of 12.75 s (calculated as $5 \times T_1$) was applied between acquisitions for fully relaxed ^1H -NMR spectra. The external standard TMSP was used as a chemical shift reference (0 ppm).

All spectral acquisition and data analysis were performed using the Bruker TopSpin software. Cell metabolites were identified based on the results from our chemical shift data base and/ or referred to the Human Metabolome Database from the University of Alberta (<http://www.hmdb.ca/>). After performing Fourier transformation, the absolute concentrations of 75 identified endogenous

metabolites were then referred to the TMSP integral and calculated as [micromol/g] according to the equation (7):

$$C_x = \frac{I_x : N_x \times C}{I : 9} \times V : M_{sample} \quad (2)$$

where C_x = metabolite concentration

I_x = integral of endogenous metabolite ^1H peak

N_x = number of protons in metabolite ^1H peak (from CH , CH_2 , CH_3 , etc.)

C = TMSP concentration

I = integral of TMSP ^1H peak at 0 ppm (:9 since TMSP has 9 protons)

V = total volume of the sample with D_2O (0.02 mL)

M_{sample} = volume of cell sample (g)

ELISA for Cytokine Levels

For BM aspirates, the femur and tibias of mice were collected, weights normalized, and BM cells were liberated as previously described (8). The cell preparation was then centrifuged at 2000 rpm for 10 minutes, and 1 mL of BM aspirate (supernatant) was collected in 1.5 mL eppendorf tubes. For serum, blood was isolated from mice using cardiac puncture (23 gauge needle with at 1 mL syringe) and transferred to a 1.5 mL Eppendorf tube. The tubes were left undisturbed at room temperature for 30 minutes. Samples were then centrifuged at 3000 rpm for 10 minutes, and serum (supernatant) was collected, and stored at -20°C until processing. ELISA analysis for $\text{TNF-}\alpha$ and IL-6 was performed per the

manufacturer's protocols (BD OptEIA; Mouse TNF ELISA Kit, Cat. No. 560478/ Mouse IL-6 ELISA Kit, Cat. No. 555240 and R & D Systems; IL-1 β ELISA Kit, Cat. No. DY201).

Meso Scale Discovery (MSD) Assays

Pro- and Pre-B cell progenitors were sorted from mice, and the levels of total STAT5a/b and phosphorylated-STAT5a protein was determined using MSD assays per the manufacturer's protocol (MSD; phospho (Tyr694)/Total STAT5a,b Whole Cell Lysate, cat. no. K15163D-2). Briefly, MSD plates were blocked with blocking buffer while shaking on an orbital shaker for 1 hour at room temperature. The plates were washed three times, and samples were added to the plate at a concentration of 10 μ g/ well of whole cell lysate. The plates were incubated (while shaking) for 1 hour at room temperature. The plate was again washed 3 times, the detection antibody was added, and the plate was incubated (while shaking) for 1 hour at room temperature. After incubating, the plate was washed 3 times and 1X Read Buffer T was added to each well of the MSD plate. The plates were analyzed on the SECTOR Imager (Meso Scale Discovery).

ATP and NADH Assays

ATP levels in B-progenitor cells were detected using the CellTiter-Glo Luminescent Cell Viability Assay (Promega; Cat. No. G7570) per protocol instructions. Briefly, 10⁵ cells were plated in 100 μ l of 10% RPMI in opaque-walled, 96-well plates and plates were allowed to equilibrate for 30 minutes at room temperature. After equilibration, 100 μ l of CellTiter-Glo Reagent was added to each well and the contents of the plate were mixed for 2 minutes on an orbital shaker to induce lysis. The plates were then incubated at room temperature to stabilize the luminescent signal. After incubation, the luminescence signal was recorded using the GloMax-Multi Detection System (Promega).

NADH levels in B-progenitor cells were detected using CellTiter 96 AQueous One Solution Reagent (Promega; Cat. No. G3582) per protocol instructions. Briefly, 10^5 cells were plated in 100 μ l of 10% RPMI in opaque-walled, 96-well plates and 20 μ l of CellTiter 96 AQueous One Solution Reagent was pipetted into each well. Plates were then incubated at 37°C for 1 hour in a humidified, 5% CO₂ incubator. After the 1 hour incubation, the absorbance at 490nm was recorded using the GloMax-Multi Detection System (Promega).

Mitochondrial ROS Detection

Mitochondrial superoxide was detected in B-progenitors isolated from young (2 mo) and old mice (24 mo) mice using the MitoSOX Red Mitochondrial Superoxide Indicator (Life Technologies; catalog no. M36008). Bone marrow cells were plated at a concentration of 10^5 cells/ well in 96 well round bottom plates, and incubated in the dark on ice for 30 minutes with anti- B220-APC antibodies in order to identify B-cell progenitor populations (BD Pharmingen; catalog no. 553092). After incubation, the cells were pelleted by centrifugation (1000 rpm for 4 minutes), and the supernatant was removed from each well. The MitoSOX Red Mitochondrial Superoxide working solution was then added to each well (100 μ l/ well), and the plate was incubated at 37°C for 10 minutes. Cells were then pelleted and the supernatant removed as described above, and the cells were washed 2 times with 10% RPMI prior to determining superoxide levels using the Cyan ADP Analyzer (Beckman Coulter).

Apoptosis Assays

Bone marrow cells from young (5 mo) and old mice (20 mo) were plated at a concentration of 10^5 cells/ well in a 96 well round bottom plate, and surface stained with anti-B220-APC and anti-CD43-FITC in order to identify B-cell progenitor populations (BD Pharmingen; catalog nos. 553092 and 553270,

respectively). After incubation, the cells were pelleted by centrifugation (1000 rpm for 4 minutes), and the supernatant was removed from each well. Cells were then resuspended in 100 μ l of 1X PBS, and 100 μ l of Millipore Guava Nexin Reagent (Millipore; part no. 4700-0450) was added to each well. The plate was then incubated in the dark at room temperature for 20 minutes. Samples were then acquired on the Cyan ADP Analyzer (Beckman Coulter).

Cell Lines

Ba/F3 cells (pro B-cell line) were obtained from the Cancer Center cell line depository. Ba/F3 cells were transduced with MSCV retrovirus expressing the indicated oncogenes (together with GFP), and then cell sorted to obtain GFP⁺ populations for analyses. Prior to experimentation, cells were tested to ensure that they were free of mycoplasma contamination using the e-Myco PLUS Mycoplasma PCR Detection Kit (Boca Scientific, catalog no. 25237).

Cultured Cell Experiment for ELISA

Bone marrow cells were harvested from young (5 months) and old (22 months) C57BL/6 mice. MACs selection was performed per protocol instructions in order to isolate B-cell progenitors and macrophages using microbeads specific for CD45R (Miltenyi Biotec, cat. no. 130-049-501) and CD11b (Miltenyi Biotec, cat. no. 130-049-601). Purified B-cell progenitors (CD45R⁺) were plated at a density of 5×10^5 cells/ well in 12-well plates with 10% RPMI media supplemented with cytokines (IL-7 and Flt3, both at 10 ng/ mL). Macrophages (CD11b⁺) were plated as described above using 10% RPMI media supplemented with GM-CSF (10 ng/ mL). Cells were cultured for 24 hours, supernatants were harvested,

and ELISAs for IL-6 and IL-1 β were performed using the protocols and kits listed in the “ELISA for Cytokine Levels” section above.

For bone marrow stromal cells: 6 x 10⁵ bone marrow cells were plated in 12-well plates with 10% RPMI and cultured for 4 hours at 37° in order to allow adherent cells to attach to the plate. After incubation, non-adherent cells were then removed by gently pipetting off the supernatant. Cells were cultured for 24 hours, and ELISAs for IL-6 and IL-1 β were performed as described above.

Additionally, adherent cells were trypsinized, and stained with antibodies in order to determine the number of bone marrow stromal cells that adhered to the plate (BM stromal cells were identified using flow cytometry based on their negative surface stain for the lineage markers [B220/ CD3/ CD11b/ CD11] and their positive expression of CD200 [eBioscience, catalog no. 12-5200-80]). These cultures resulted in 85% of adherent cells having a bone marrow stromal cell phenotype. Based on this percentage, we determined that 5 x 10⁵ bone marrow stromal cells were cultured overnight (which was equivalent to the number of B-cell progenitors and macrophages that were cultured in similar experiments).

REFERENCES

1. Irizarry, R.A., Bolstad, B.M., Collin, F., Cope, L.M., Hobbs, B., and Speed, T.P. 2003. Summaries of Affymetrix GeneChip probe level data. *Nucleic Acids Res* 31:e15.
2. Subramanian, A., Tamayo, P., Mootha, V.K., Mukherjee, S., Ebert, B.L., Gillette, M.A., Paulovich, A., Pomeroy, S.L., Golub, T.R., Lander, E.S., et al. 2005. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proc Natl Acad Sci U S A* 102:15545-15550.
3. Kanehisa, M., Goto, S., Furumichi, M., Tanabe, M., and Hirakawa, M. 2010. KEGG for representation and analysis of molecular networks involving diseases and drugs. *Nucleic Acids Res* 38:D355-360.
4. Fleenor, C.J., Rozhok, A.I., Zaberezhnyy, V., Mathew, D., Kim, J., Tan, A.C., Bernstein, I.D., and DeGregori, J. 2015. Contrasting Roles for C/EBPalpha and Notch in Irradiation-Induced Multipotent Hematopoietic Progenitor Cell Defects. *Stem Cells* 33:1345-1358.
5. Klawitter, J., Schmitz, V., Leibfritz, D., and Christians, U. 2007. Development and validation of an assay for the quantification of 11 nucleotides using LC/LC-electrospray ionization-MS. *Anal Biochem* 365:230-239.
6. Serkova, N.J., and Glunde, K. 2009. Metabolomics of cancer. *Methods Mol Biol* 520:273-295.

7. Serkova, N.J., Jackman, M., Brown, J.L., Liu, T., Hirose, R., Roberts, J.P., Maher, J.J., and Niemann, C.U. 2006. Metabolic profiling of livers and blood from obese Zucker rats. *J Hepatol* 44:956-962.
8. Henry, C.J., Marusyk, A., Zaberezhnyy, V., Adane, B., and DeGregori, J. 2010. Declining lymphoid progenitor fitness promotes aging-associated leukemogenesis. *Proc Natl Acad Sci U S A* 107:21713-21718.

Fig. S1

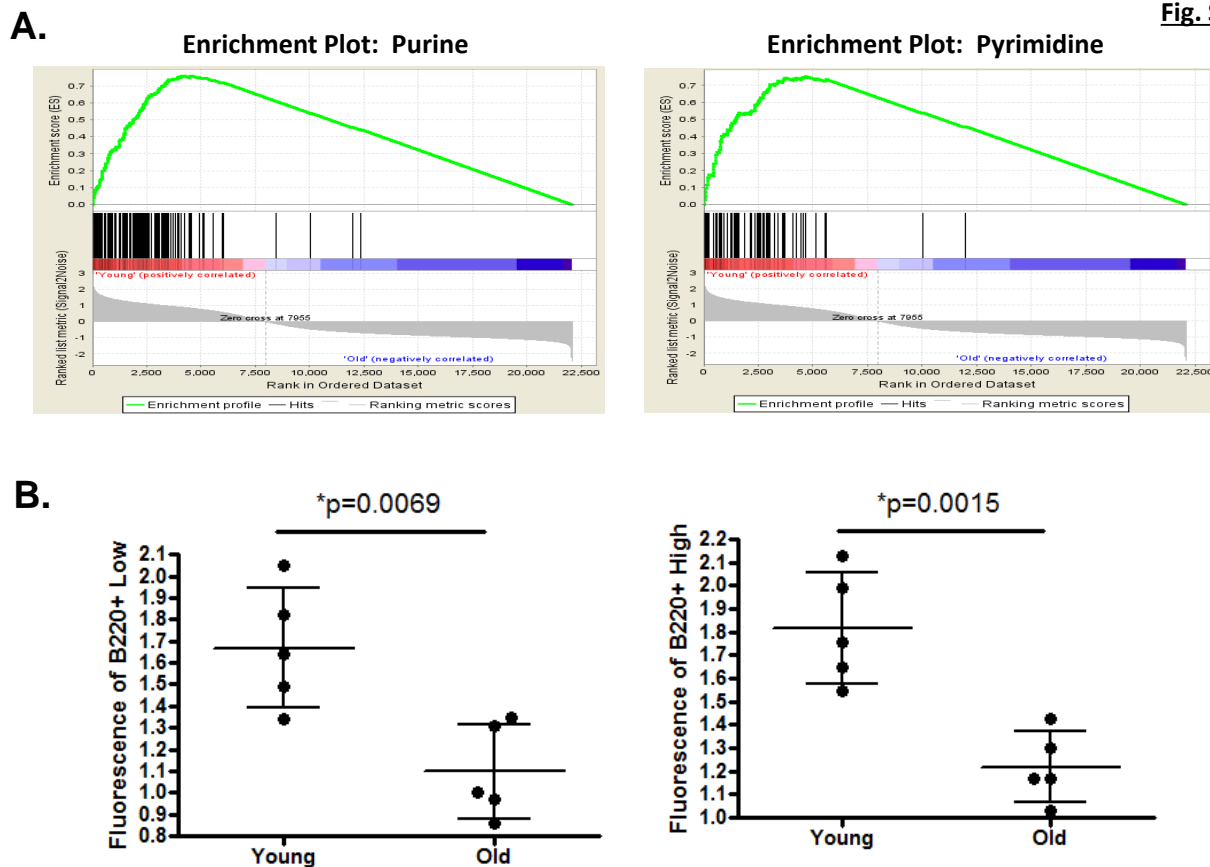


Figure S1. Aging-associated Declines in Nucleotide Anabolism and Metabolism Accompanies Aging B-lymphopoiesis

(A) Purine and pyrimidine pathway enrichment plots by GSEA for microarray gene expression analyses of B-progenitors isolated from young (Y) and old (O) BALB/c mice (2 and 24 months; 3 mice/ age group). **(B)** Mitochondrial superoxide levels was determined in B-progenitors isolated from young and old C57BL/6 mice (2 and 24 months, respectively) using the MitoSOX Red Mitochondrial Superoxide Indicator. Values in **B** represent mean \pm SEM with 5 mice per age group. Statistical analyses were performed using Student's t-test relative to the young control.

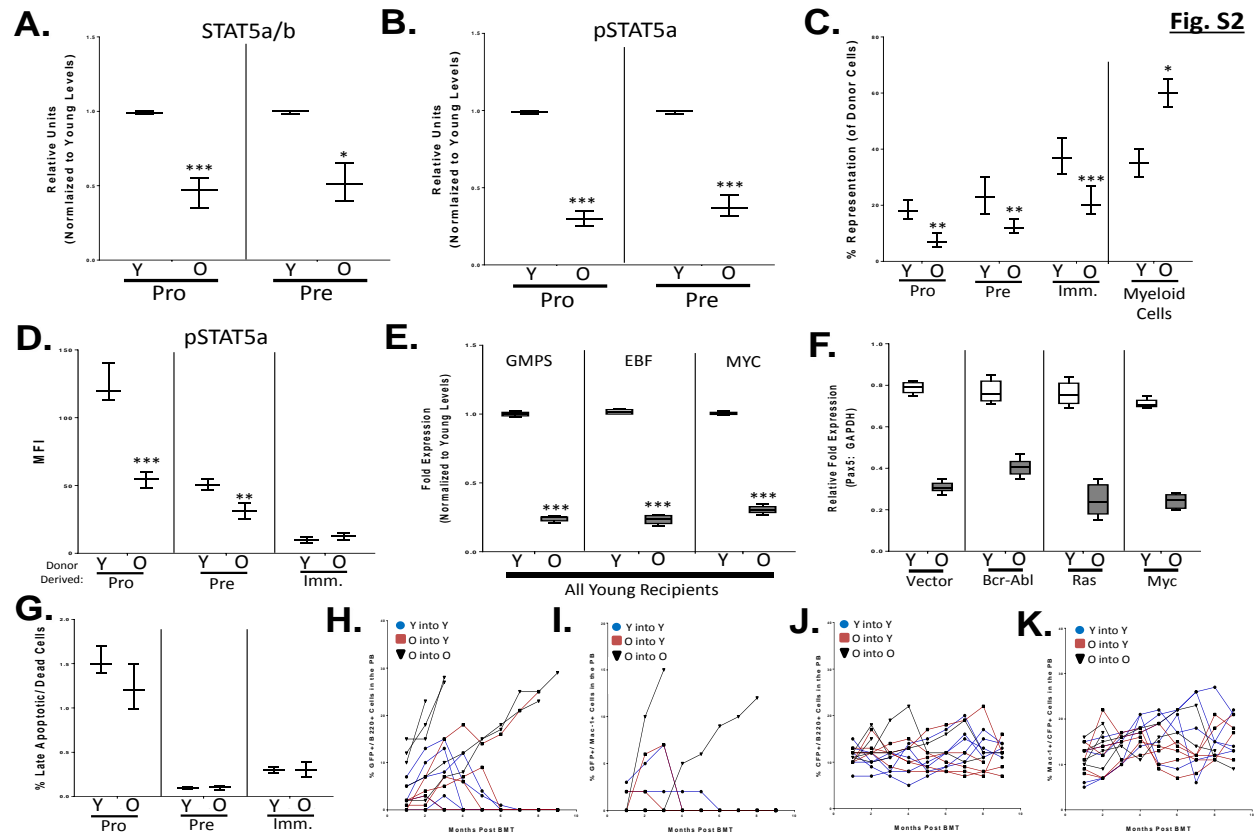


Figure S2. A Young Microenvironment Cannot Reverse Aging-associated Functional Defects in B-progenitors

Pro- and Pre-B cell progenitors were sorted from young (2 month) and old (22 month) mice, and the total **(A)** and phosphorylated **(B)** levels of STAT5a/b protein were determined using MSD analysis. Values in **(A and B)** represent mean \pm SEM with 3 donor mice per age group. **(C-E)** BM cells from young (2 month) and old (24 month) GFP⁺ Balb/c donor mice were transplanted into young GFP⁺ Balb/c recipient mice. At 4 months post-transplantation, the contribution of donor derived cells to hematopoiesis was determined using flow cytometry **(C)**. Donor-derived B-progenitors were sorted from recipient mice, and the levels of phosphorylated-STAT5 in these populations were determined using flow cytometry **(D)**. The expression levels of *Gmps*, *Ebf* and *Myc* in donor-derived sorted pro-B cells were determined using qRT-PCR analysis **(E)**. Values in **(C-E)** represent mean \pm SEM with 3 donor mice per age group. **(F)** *Pax5* gene expression was determined using qRT-PCR in vector expressing or oncogene-expressing pro-B cell progenitors (described in Figures 2D-2F). Values represent mean \pm SEM for >5 mice per group. **(G)** The percentage of apoptotic B-progenitor populations isolated from young (5 months) and old (20 months) mice was determined using the Millipore Guava Nexin Reagent Kit. As a positive control, BM cells were treated with 0.5mM H₂O₂ for 1 hour, and 47%, 32%, and 18% of pro-B, pre-B, and immature B cells respectively were found to be apoptotic. Values represent approximately 3 mice per age group. All statistical analyses were performed using Student's t-test relative to the young control for each experiment. **(H-K)** The percentage of *NRAS*-expressing (GFP⁺) or vector-expressing (CFP⁺) B-cells and macrophages were tracked in the peripheral blood of mice in the experiment described in **Figure 2I**.

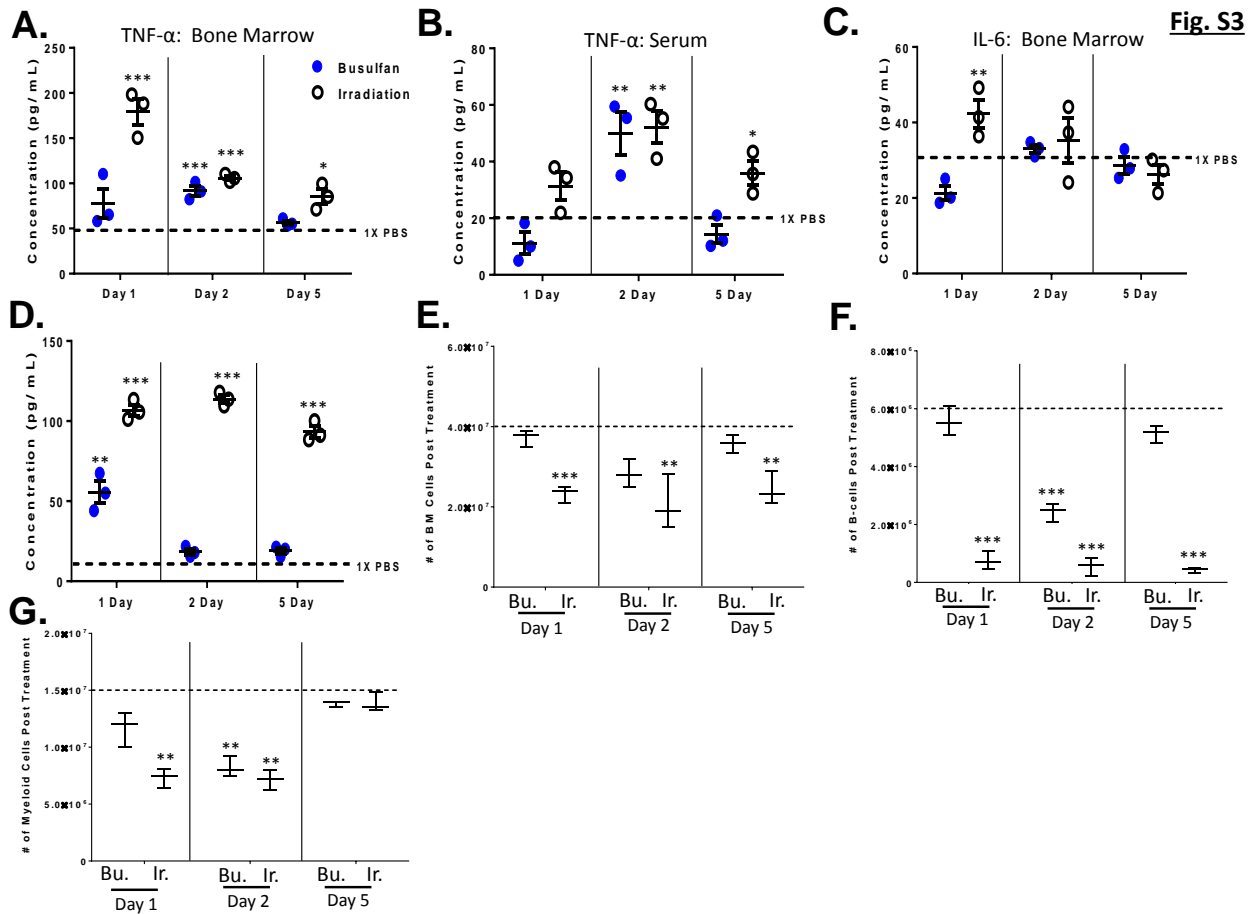


Figure S3. Busulfan Conditioning Reduces Experimentally Induced Inflammation

(A-D) C57BL/6 mice were sublethally irradiated or treated with one dose of busulfan (25 mg/kg), and cytokine levels in BM aspirates and serum was determined at the indicated time points post treatment via ELISA analysis. (E-G) The representation of various hematopoietic populations from mice treated in (A-D) was determined at various time points post treatment using flow cytometric analysis. Values represent mean \pm SEM with 3 mice per group per timepoint. All statistical analysis was performed using Student's t-test relative to the vehicle (1X PBS).

Fig. S4

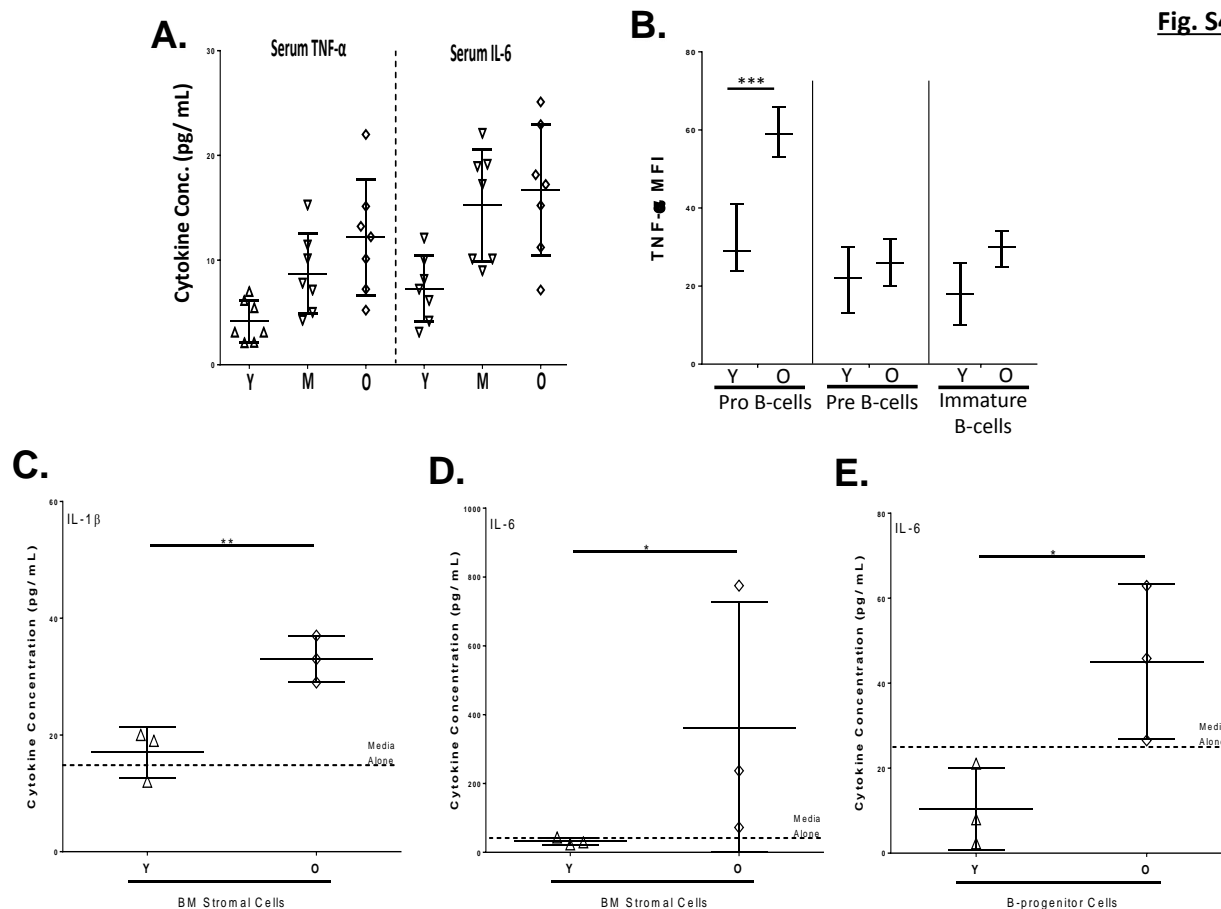


Figure S4. Inflammatory Mediators Increase with Age in Mice

(A) Serum was collected from young (2 months), middle-aged (14 months), and old (24 months) mice and the levels of TNF- α and IL-6 were determined using ELISA. Values represent mean \pm SEM from 2 independent experiments with >6 mice per age group. **(B)** Bone marrow cells were harvested from young (5 months) and old (20 months) mice and stained to identify B-progenitor populations producing TNF- α using flow cytometric approaches. Values represent approximately 3 mice per age group. **(C-E)** Bone marrow stromal cells or B-cells progenitors from young (5 months) and old (22 months) mice were cultured overnight, and ELISA analysis for IL-1 β and IL-6 was performed on the supernatants. Values represent mean \pm SEM from 3 mice per age group. For each experiment, statistical analysis was performed using Student's t-test relative to the young control.

Fig. S5

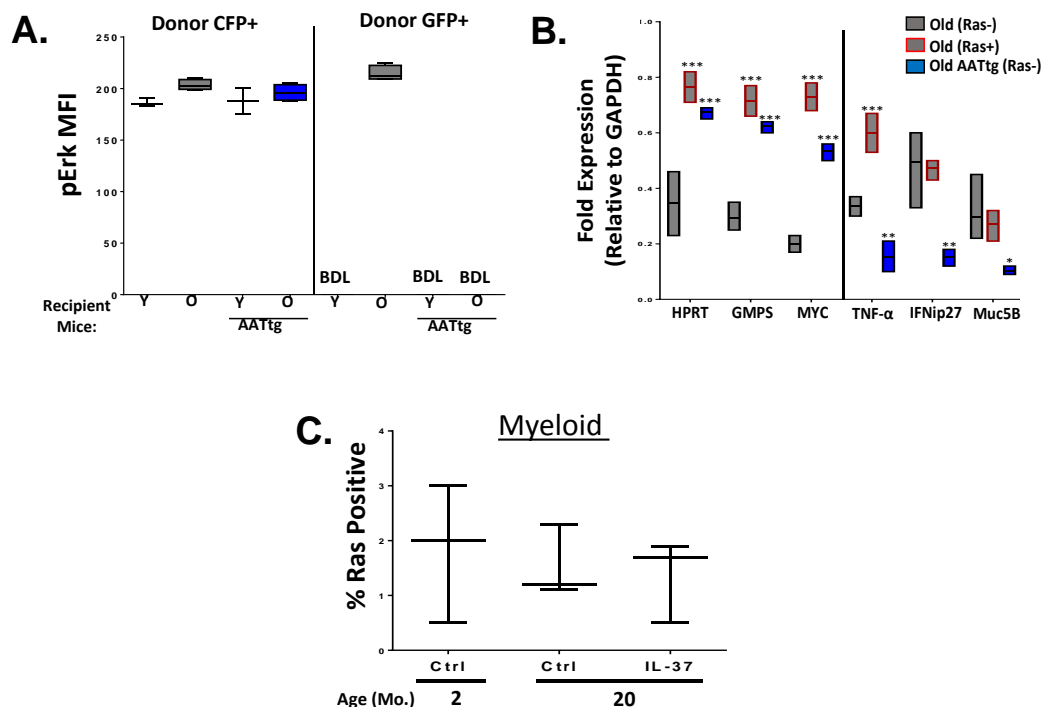


Figure S5. The Impact of the BM Microenvironment on Signaling, Gene Expression, and Oncogenic Selection.

Young (2 months) and old (20 months) littermate or AAT transgenic mice were transplanted with $NRAS^{V12}$ -expressing cells as in Figure 7. At 2 month post-transplantation, **(A)** Erk activation was analyzed in donor pro-B cells (CD45.1⁺) expressing vector (CFP⁺) or $NRAS^{V12}$ (GFP⁺) isolated from young and old LC and AAT transgenic recipients using flow cytometry. **(B)** The expression levels of the indicated genes in donor pro B-cells expressing vector (V) or $NRAS^{V12}$ (RAS) were determined via qRT-PCR. **(C)** Young (2 months) mice and old (20 months) littermate or IL-37 transgenic mice were transplanted with $NRAS^{V12}$ -expressing cells. At 3 month post-transplantation, BM was analyzed for the representation of $NRAS^{V12}$ -expressing myeloid cells using flow cytometry. Values in **(A and B)** represent mean \pm SEM with >5 mice per group. All statistical analysis was performed using Student's t-test relative to the young control or non-oncogenic cells from aged recipient mice (S5B, black-outlined-gray bars).