Aging-associated inflammation promotes selection for adaptive oncogenic events in B cell progenitors

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The incidence of cancer is higher in the elderly; however, many of the underlying mechanisms for this association remain unexplored. Here, we have shown that B cell progenitors in old mice exhibit marked signaling, gene expression, and metabolic defects. Moreover, B cell progenitors that developed from hematopoietic stem cells (HSCs) transferred from young mice into aged animals exhibited similar fitness defects. We further demonstrated that ectopic expression of the oncogenes BCR-ABL, NRASV12, or Myc restored B cell progenitor fitness, leading to selection for oncogenically initiated cells and leukemogenesis specifically in the context of an aged hematopoietic system. Aging was associated with increased inflammation in the BM microenvironment, and induction of inflammation in young mice phenocopied aging-associated B lymphopoiesis. Conversely, a reduction of inflammation in aged mice via transgenic expression of α-1-antitrypsin or IL-37 preserved the function of B cell progenitors and prevented NRASV12-mediated oncogenesis. We conclude that chronic inflammatory microenvironments in old age lead to reductions in the fitness of B cell progenitor populations. This reduced progenitor pool fitness engenders selection for cells harboring oncogenic mutations, in part due to their ability to correct aging-associated functional defects. Thus, modulation of inflammation—a common feature of aging—has the potential to limit aging-associated oncogenesis.

Introduction

The incidence of many human diseases, including cancers, increases with age (1). The aging process is associated with functional impairment at the tissue, cellular, and molecular levels (2). Aging-associated changes are particularly evident in the BM of mice and humans, manifesting as reductions in BM density, changes in vascularization, and alterations in the composition of accessory and structural cells (3).

The function of hematopoietic stem and progenitor cells (HSPCs) significantly declines with age in both mice and humans (4–7). In competitive transplantation assays, old hematopoietic stem cells (HSCs) exhibit reduced reconstitution potential on a per-cell basis (a measure of “cellular fitness”) when compared with HSCs isolated from young mice. Aged HSCs also exhibit altered metabolism and localization in the BM as well as reduced BM homing potential upon transplantation (8–10). Aging-associated hematopoietic defects are particularly apparent in the lymphoid lineages, which in mice and humans can be partly attributed to the skewing of the HSC repertoire toward myeloid-biased HSCs (6, 10–13). Additional studies have indicated that the aged BM microenvironment can contribute to reductions in B lymphopoiesis (14, 15). Notably, we have previously shown that declining B lymphoid progenitor fitness in old mice promotes selection for the BCR-ABL oncogene due to its ability to correct aging-associated defects in cell signaling (16).

More recently, it has been demonstrated that inflammatory cytokines regulate the function of hematopoietic progenitor cells. TNF-α and TGF-β (at high concentrations) have been shown to suppress HSC activity (17, 18), whereas IFN-α, IFN-γ, and TGF-β (at low concentrations) activate HSC proliferation (19–21). Moreover, inflammation has been shown to directly impair B lymphopoiesis (and thus favor myelopoiesis) by preventing B progenitor localization to the IL-7–rich niches required for B cell development (22–24). In aged mice, the production of TNF-α by aging-associated B cells impairs B lymphopoiesis (14, 25), and the myeloid-biasing of the hematopoietic compartment with age is in part regulated through the actions of TGF-β1 (18). Whereas inflammation is important for survival in youth to combat infections and repair tissues, it can have adverse effects on aged individuals (26, 27). Indeed, older humans typically present a subclinical systemic chronic inflammatory status termed “inflamm-aging,” which has been postulated to contribute to the development of a variety of aging-associated diseases such as Alzheimer’s disease, cardiovascular disease, and cancer (28–31).

Current paradigms attribute the association between aging and cancer primarily to the progressive accrual of oncogenic mutations that are widely thought to be the rate-limiting events in the generation of most cancers (32–34). Predominant models of carcinogenesis mostly assume that mutations convey defined fitness effects on transformed cells; however, this idea contra-
defined as a measure of the ability of stem/progenitor cells of a certain epigenotype genotype to transmit this type to subsequent cell generations.

Given the strong correlations between advanced age, chronic systemic inflammation, and cancer incidence in mammals, in this study we sought to determine how aging-associated inflammation impacts lymphoid progenitor populations and how this state influences the evolution of leukemias. Using transgenic expression of two different proteins, α-1-antitrypsin (AAT) and IL-37, in order to reduce inflammation in old mice, we show that preventing aging-associated reductions in B progenitor fitness abrogates selection for oncogene-initiated progenitors.

dicts evolutionary theory, which holds that fitness is dictated by the interaction of a genotype-defined phenotype with the environment (35). Similarly, the somatic mutation theory of aging largely attributes age-dependent tissue decline to the accumulation of somatic mutations throughout life (2, 32, 33, 36). Our laboratory has computationally modeled fitness changes and somatic evolution in HSC pools during life to demonstrate that mutation accumulation alone cannot account for either HSC fitness decline or late-life clonal evolution (35). Importantly, these modeling studies demonstrate that age-dependent alterations in the tissue microenvironment are necessary for both HSC fitness decline and clonal evolution (where cellular “fitness” is defined as a measure of the ability of stem/progenitor cells of a certain epigenotype genotype to transmit this type to subsequent cell generations).

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Results

Metabolic and cell-cycle defects accompany aging B lymphopoiesis.

In order to understand the mechanism underlying declining B lymphopoiesis in old age, we performed microarray analysis on combined pro- and pre-B cell populations isolated from young (2-month-old) and old (24-month-old) mice. Gene set enrichment analysis (GSEA) revealed that aging B lymphopoiesis is accompanied by significant reductions in purine and pyrimidine metabolism (Figure 1A and Supplemental Figure 1A), suggesting that metabolic changes also accompany aging B lymphopoiesis. Indeed, nuclear magnetic resonance (NMR) analysis revealed significant decreases in total nucleotide and adenosine levels in aged B cell progenitors (Figure 1D), consistent with reduced purine synthesis resulting from decreased expression of genes such as Hprt and Gmps. Furthermore, other metabolic intermediates such as citrate (a key TCA cycle intermediate) and glutamine (an amino acid that can enter the TCA cycle) were also significantly downregulated in old B progenitors (Figure 1D). Importantly, lactic acid (a marker for increased glycolytic output and mitochondrial impairment) was also found to be increased in old B cell progenitors (Figure 1D). These metabolic perturbations coincided with significant reductions in ATP, reduced ATP/ADP and GTP/GDP ratios, elevated NADH levels, and significant reductions in mitochondrial ROS levels in aged B cell progenitors (Figure 1D). Notably, IL-7R signaling also significantly decreased Hprt gene expression in pro-B cells (Figure 1C), suggesting that aging-associated reductions in purine synthesis gene expression can be explained, at least in part, by an impairment of IL-7R signaling.

Our microarray analysis also indicated that mitochondrial dysfunction accompanied aging B lymphopoiesis (P = 7.01 × 10^-7 for mitochondrial dysfunction using Ingenuity Pathway Analysis [IPA]), suggesting that metabolic changes also accompany aging B lymphopoiesis. Indeed, nuclear magnetic resonance (NMR) analysis revealed significant decreases in total nucleotide and adenosine levels in aged B cell progenitors (Figure 1D), consistent with reduced purine synthesis resulting from decreased expression of genes such as Hprt and Gmps. Furthermore, other metabolic intermediates such as citrate (a key TCA cycle intermediate) and glutamine (an amino acid that can enter the TCA cycle) were also significantly downregulated in old B progenitors (Figure 1D). Importantly, lactic acid (a marker for increased glycolytic output and mitochondrial impairment) was also found to be increased in old B cell progenitors (Figure 1D). These metabolic perturbations coincided with significant reductions in ATP, reduced ATP/ADP and GTP/GDP ratios, elevated NADH levels, and significant reductions in mitochondrial ROS levels in aged B cell progenitors (Figure 1D). Notably, impairments in B cell production, STAT5-mediated signaling, and the expression of key genes involved in purine synthesis (Gmps), B cell development (Ebf), and proliferation (Myc) were not reversed by transplantation into irradiated young mice (Supplemental Figure 2, C–E). These observations indicate that aging-associated reductions in IL-7R signaling in B cell progenitors result, at least in part, from the reduced availability of key signaling intermediates, and a young nonhematopoietic microenvironment cannot correct aging-associated defects in B cell progenitors that develop from the transplanted old HSC.

In order to study oncogenic adaptation in vivo, hematopoietic progenitor cells from young and old mice were retrovirally transduced to introduce the oncogenes BCR-ABL, NRASV12, or Myc and transplanted into lethally irradiated young recipient mice. Three weeks after transplantation, oncogene or vector-bearing pro-B cell progenitors (GFp+) were flow sorted from recipient mice and their signaling and gene expression profiles determined. The expression of oncogenic BCR-ABL, NRASV12, and Myc each led to restoration of p-STAT5 activation in pro-B cells (Supplemental Figure 2C). In contrast, expression of these oncogenes did not lead to significant alterations in p-STAT5 levels in young pro-B cells, for which signaling was already high. We also observed that oncogenic BCR-ABL, NRASV12, and Myc restored youthful expression of key signaling intermediates, and a young nonhematopoietic microenvironment cannot correct aging-associated defects in B cell progenitors that develop from the transplanted old HSC.

Oncogenic mutations correct aging-associated functional defects in B progenitors. Given that BCR-ABL expression corrects defective IL-7R-mediated signaling in aged B cell progenitors, leading to increased leukemogenesis in aged backgrounds (16), we determined whether BCR-ABL expression reverses aging-associated defects in key genes involved in metabolism and DNA replication. Since BCR-ABL expression activates RAS and MYC (41), we also asked whether the NRASV12 and MYC oncogenes are adaptive in aged backgrounds by correcting aging-associated B progenitor fitness defects.

Using the Ba/F3 pro-B cell line, we found that directly inhibiting cytokine receptor signaling on B lineage cells in vitro was sufficient to reduce the expression of key nucleotide synthesis genes, as reducing IL-3-mediated stimulation of the Ba/F3 pro-B cell line impaired STAT5 phosphorylation (Figure 2A) and expression of Myc (Figure 2B) and Hprt (Figure 2B). Importantly, the expression of oncogenic BCR-ABL, NRASV12, and Myc maintained STAT5 phosphorylation and Myc and Hprt expression levels in Ba/F3 cells despite cytokine withdrawal (Figure 2, A and B), indicating a direct ability of these oncogenes to rescue cytokine receptor signaling defects.

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Given the extensive metabolic changes associated with aging B lymphopoiesis as well as the findings of previous studies demonstrating increased replicative stress and cell-cycle defects in HSCs from old mice (39), we determined the consequences of these perturbations on cell-cycle progression. Mice of various ages were injected with the nucleotide analog 5-ethyl-2′-deoxyuridine (EdU). We observed significant reductions in B lymphopoiesis in mice between 14 and 22 months of age, as indicated by a decrease in the ratio of B cell (B220+) to myeloid cell (MAC1+) progenitors (Figure 1H). Notably, aged pro-B cell progenitors exhibited a significant reduction in the rate of S-phase progression (Figure 1, I and J); however, we did not observe increased apoptosis in aged B progenitor populations (Supplemental Figure 2G). Overall, these results reveal that aging B lymphopoiesis is accompanied by extensive metabolic changes that culminate in defective cell-cycle progression in B cell progenitors. Since these alterations coincide with the reduced competitive potential of old B progenitors (40) and impairing IL-7R signaling is sufficient to reduce the function of B cell progenitors and impair B lymphopoiesis, we conclude that these signaling, metabolic, and cell-cycle defects are contributing to an age-associated decline in B progenitor fitness.
levels of Hprt (Figure 2D) and endogenous Myc (Figure 2E). Interestingly, the defective expression of the B lineage specification genes Ebf (Figure 2F) and Pax5 (Supplemental Figure 2F) in aged B cell progenitors was not restored by these oncogenes. From these results, we conclude that BCR-ABL, NRASV12, and Myc expression restores the key parameters of fitness, but not the differentiation potential, of aged B progenitors.

We next determined whether NRASV12- and Myc-mediated restoration of impaired fitness in aged B progenitors, by providing a competitive advantage within poorly fit old B progenitor pools, promotes increased leukemogenesis. Lethally irradiated young mice were prepopulated with young or old BM cells to create young and aged hematopoietic systems, respectively, and then transplanted with either young or old BM progenitor cells transduced with the NRASV12 and Myc oncogenes. Oncogene-driven restoration of B progenitor function in old, but not young, pro-B cells led to increased NRASV12- and Myc-mediated leukemogenesis in aged backgrounds. In contrast, leukemogenesis was largely
suppressed in young hematopoietic contexts (Figure 2, G and H). Thus, provision of the oncogenic drivers is insufficient to induce leukemias, as the leukemic potential of the oncogenic mutations is only fully realized in the aged hematopoietic context. Notably, we have previously shown that the differential ability of BCR-ABL to induce leukemias in young and old hematopoietic backgrounds does not require an intact adaptive immune system (16).

We then asked whether the age of the host would similarly impact leukemogenesis; specifically, would transplantation of NRASV12-transduced old BM into a young recipient still sufficiently induce leukemias? In order to reduce the transplant-induced affects on the host BM microenvironment, we used busulfan rather than γ irradiation to condition recipient mice prior to transplantation with NRASV12-transduced BM. Busulfan conditioning induced a mild inflammatory response, as indicated by transient increases in BM and serum IL-6 and TNF-α levels, which returned to baseline by day 5 after treatment (Supplemental Figure 3, A–D). BM hematopoietic cell populations returned to homeostatic numbers by day 5 after busulfan treatment (Supplemental Figure 3, E–G). In contrast, sublethal irradiation induced greater and more prolonged increases in these cytokines, and B lymphopoiesis was still highly suppressed on day 5 after irradiation (Supplemental Figure 3, A–G). Importantly, transplantation of oncogenic NRASV12-transduced old BM progenitors into young mice resulted in significantly less leukemia than their transplantation into old recipient mice (Figure 2I). We tracked the frequencies of NRASV12-expressing (GFP+) B cells and myeloid cells in peripheral blood over the course of the experiment and found that the percentage of B lineage cells expressing NRASV12 was only maintained and amplified in old recipient mice, but not in young recipient mice, even when donor progenitor cells were old (Supplemental Figure 2, H–K). Thus, the age of the microenvironment may be more important than the age of the cells receiving the oncogenic hit in leukemia development. In total, these results reveal that oncogenic BCR-ABL, NRASV12, and Myc expression correct functional defects in aged pro–B cells, leading to increased selection in aged hematopoietic backgrounds, with subsequent progression to B-lineage leukemias.

Increased inflammation in the BM with age coincides with decreased expression of genes regulating cell-cycle progression in B progenitors. Since inflammation increases with age in most mammals (31) and regulates the function of HSPCs (42), we explored the relationship between aging-associated increases in inflammation and B lymphopoiesis. Principal component analysis (PCA) of the microarray data indicated that gene expression profiles for B progenitors isolated from individual young mice exhibited low intra-group variability; in contrast, B progenitors isolated from old mice exhibited divergent gene expression profiles (Figure 3A). Notably, reductions in E2F and MYC target genes, including genes involved in nucleotide synthesis and cell-cycle progression, coincided with increases in the inflammatory signature (Figure 3B). In order to determine the kinetics of altered inflammation with age, we performed ELISAs for inflammatory cytokines on serum and BM conditioning media from young versus old mice. Cytokines were consistently and significantly increased in the BM and serum of old mice (Figure 3, C and D). Furthermore, both IPA (Figure 3E) and GSEA (Figure 3F) revealed that the expression of TNF-α and its target genes was significantly increased in aged B cell progenitor populations (P = 4.23 × 10−3 for TNF-α stimulatory pathways using IPA). These findings were corroborated by flow cytometric analyses, which revealed that the production of TNF-α significantly increased with age in pro–B cell progenitors (Supplemental Figure 4B). In addition, BM stromal cell cultures from old mice exhibited elevated production of IL-1β and IL-6 relative to the levels detected in cultures from young mice, while BM B cell lineage cultures from old mice only exhibited increased IL-6 production (Supplemental Figure 4, C–E). In addition to increased Tnfa mRNA expression, Ifi36 and IFN-α-inducible protein 27 (Ifi27) mRNA expression levels were also evident in sorted pro–B cell progenitors isolated from old mice (Figure 3G). Similarly, Muc5b, which has been shown to be activated in a variety of cell types in response to inflammation (43), was only found to be expressed in aged, but not young, pro–B cell progenitors. These experiments revealed that aging is associated with increased inflammation in the BM and within resident B cell progenitors. Contrasting with the correction of aging-associated fitness defects in pro–B cells (Figure 2), the expression of BCR-ABL and NRASV12 significantly increased the expression of Tnfa in aged pro–B cell progenitors (Figure 3H). Notably, the expression of BCR-ABL, NRASV12, or Myc did not elicit TNF-α production from young pro–B cell progenitors (Figure 3H). Thus, oncogene expression can actually exacerbate the expression of inflammatory cytokines specifically in old progenitors.

Ectopically promoting inflammation in young mice phenocopies aging-associated B lymphopoiesis. We asked whether increasing inflammation is sufficient to impair B cell progenitor fitness. To this end, young mice were injected with LPS or recombinant TNF-α every 4 days for a period of 2 weeks. As a control, we injected all-7 to impair B lymphopoiesis. After 2 weeks of treatment, we observed significant reductions in the percentages of pro–, pre–, and immature B cells in the BM of mice treated with all-7 or inflammatory mediators (LPS or TNF-α) (Figure 4A), consistent with previous results (22–24). Importantly, acute inflammation also significantly decreased expression levels of the B lineage specification genes Pax5 and Ebf3 (Figure 4B) as well as expression of the purine synthesis genes Hprt and Gmps (Figure 4C) in B cell progenitors, providing molecular insight into reduced B progenitor fitness with old age. Collectively, these data demonstrate that induction of inflammation in young mice mirrors the B lymphopoiesis defects observed in old mice.

Aging in most vertebrates is commonly associated with low-grade, chronic inflammation (31). We investigated whether the chronic inflammation that we observed in old mice can underlie their B lymphopoiesis defects. We monitored B cell development in TNF-αARE mice, which have a mutation in the Tnfa 3′-UTR, removing regulatory adenylate-uridylate–rich (AU-rich) elements and leading to increased constitutive TNF-α expression. These mice exhibit heightened susceptibility to chronic inflammatory diseases such as rheumatoid arthritis and Crohn disease (44, 45). Young TNF-αARE mice had significantly (~5-fold) increased circulating TNF-α levels (Figure 4D). B lymphopoiesis was significantly reduced in young TNF-αARE mice, coinciding with an increased frequency of myeloid cells (Figure 4, E and F). Furthermore,
Figure 3. Increased inflammation in the BM with age coincides with decreased expression of genes regulating cell-cycle progression in B progenitors. (A) PCA of the microarray data was generated using the Partek Genomics Suite. (B) Gene expression profiles from the microarray analysis performed on B cell progenitors isolated from young and old mice were analyzed by GSEA for the expression of genes regulated by E2F and MYC and of those involved in inflammatory processes. (C and D) BM aspirates were collected from young (Y; 2-month-old), middle-aged (M; 14-month-old), and old (O; 24-month-old) mice, and IL-6 (C) and TNF-α (D) levels were determined using ELISA. Values represent the mean ± SEM of 2 independent experiments, with more than 6 mice per age group. (E) Network analysis of the microarray data was performed using IPA software, which identified TNF-α as an important cytokine that increases in aged B progenitors. (F) Heatmap showing a subset of the inflammatory genes shown in B. (G) Pro-B cell progenitors from young (2-month-old) and old (24-month-old) mice were sorted, and expression levels of inflammatory genes (Tnfa, Ifnz, and Ifi27) or of those regulated by inflammation (e.g., Muc5b) were determined by qPCR. (H) Expression levels of TNF-α in sorted young or old pro-B cells expressing vector or oncogenic BCR-ABL, NRASV12, or Myc (all GFP+) were determined using qPCR. Values in G and H represent the mean ± SEM of 4 independent experiments, with more than 10 mice per age group. *P < 0.001, by Student’s t test relative to young controls. ND, not determined.
chronic TNF-α exposure significantly reduced Hprt and Gmps gene expression levels in sorted pro–B cells (Figure 4G). We conclude that chronic TNF-α exposure in young mice impairs the fitness of pro–B cells in a manner similar to that observed with aging.

Reducing inflammation prevents aging-associated fitness defects in B cell progenitors. We used transgenic mice that express either human AAT or human IL-37 to determine whether either of these antiinflammatory mediators could protect against age-associated fitness reductions in B progenitors. AAT is a serum serine protease inhibitor with potent antiinflammatory activity. Transgenic expression of human AAT under the surfactant protein C promoter results in low circulating levels of AAT (46, 47). Nevertheless, mice transgenic for AAT (AATtg) exhibit a remarkable resistance to a broad spectrum of inflammatory and immunological challenges (48). IL-37 is an antiinflammatory member of the IL-1 family that broadly inhibits innate inflammation (49). IL-37 transgenic (IL-37tg) mice expressing human IL-37 are protected in models of endotoxin shock, colitis, and ischemia reperfusion injury (50, 51). Strikingly, the aging-associated increases in TNF-α, IL-6, and IL-1β levels in the BM and serum of 20-month-old mice were prevented by transgenic expression of either AAT or IL-37 (Figure 5).

We observed significant reductions in the number of pro–, pre–, and immature B cell populations in aged AATtg (Figure 6A) and IL-37tg (Figure 6D) mice that were comparable to the reductions seen in nontransgenic controls. However, pro–B cells from older AATtg or IL-37tg mice had STAT5 activation levels similar to those observed in young mice, contrasting with cells from older nontransgenic mice (Figure 6, B and E). Furthermore, AAT and IL-37 expression prevented the aging-associated reductions in Hprt, Gmps, and Myc gene expression in aged pro–B cells (Figure 6, C and F). AAT expression also decreased the proinflammatory state of aged pro–B cells, as indicated by significant decreases in the expression levels of TNF-α and IFN-β (Figure 6, G).

Figure 4. Induction of inflammation promotes functional decline in B progenitors. (A–C) Young mice were injected every 4 days for 2 weeks with PBS (vehicle), αIL-7–neutralizing Abs, LPS, or recombinant TNF-α. After 2 weeks of treatment (3 injections total), the mice were sacrificed and the percentage of B progenitors in the BM determined using flow cytometry. Expression levels of B lineage–specification genes (B) and purine synthesis genes (C) were determined using qPCR. Values in A–C represent the mean ± SEM for 5 mice per treatment group. (D–G) Serum TNF-α levels in 5-month-old TNF-αΔARE mice and their littermate controls were measured by ELISA (D), frequencies of B cell (B220+) and myeloid (MAC1+) progenitor cells in BM were determined by flow cytometry (E and F), and mRNA levels of purine synthesis genes in pro–B cells were determined by qPCR (G). Values represent the mean ± SEM of 3 independent experiments, with more than 9 mice per group. *P < 0.05, **P < 0.01, and ***P < 0.001, by Student’s t test relative to PBS-injected mice or littermate controls. LC, littermate control.
Reducing inflammation in aged backgrounds prevents selection for oncogenic NRAS<sup>V12</sup>. We next determined whether reducing inflammation in aged mice altered the selection for oncogenically initiated cells. Young and old AATtg mice and age-matched controls were injected with the same pool of young BM progenitor cells transduced with retroviral vectors expressing NRAS<sup>V12</sup> plus GFP or cyan fluorescent protein (CFP) only (Figure 7A). Thus, it is the age and inflammatory status of the host microenvironment, not the cells expressing the oncogene or vector control, that is being varied. Notably, expression of the control CFP vector by peripheral B cells was not influenced by the host’s age or genetic background and did not exhibit significant changes over time (Figure 7B), indicating that homing of transduced cells and their maintenance in the host (including potential immune rejection) were not impacted by the recipient mouse’s age or AAT transgene expression. In striking contrast, we observed clear and exclusive selection for NRAS<sup>V12</sup> expression in the B cell lineage in old recipients, but not in young or old AATtg recipients (Figure 7C), which mirrored the selection for NRAS<sup>V12</sup> expression in BM B progenitors only in the nontransgenic old recipients (Figure 7D). Thus, selection for NRAS<sup>V12</sup> expression was completely prevented in B progenitor pools in the aged antiinflammatory AATtg backgrounds, indicating that aging-related inflammation is a key driver of oncogenic adaptation in B progenitors.

STAT5 activity is reduced in host (CD45.2<sup>+</sup>) pro–B cells isolated from old mice, and this decline was prevented in host pro–B cells isolated from aged AATtg mice (Figure 7E). We asked whether the age and inflammatory status of the host had an impact on fitness parameters for B progenitors that developed from transplanted young BM (CD45.1<sup>+</sup>). Indeed, young CD45.1<sup>+</sup> pro–B cell progenitors that developed in aged recipient mice also exhibited significantly reduced STAT5 activation, but not if the recipients were old AATtg mice (Figure 7F; donor CFP<sup>+</sup> cells). Similarly, expression levels of Hprt<sup>-</sup>, Gmps<sup>-</sup>, and Stat5b were all reduced in the young donor pro–B cells that developed in aged recipient mice, but not if the recipients were AATtg or young (Figure 7G and H and Supplemental Figure 5B). Thus, these key fitness parameters for B progenitors are dictated by the age and inflammatory status of the host microenvironment. Strikingly, NRAS<sup>V12</sup> expression restored p-STAT5 activation and mRNA levels of Hprt<sup>-</sup>, Gmps<sup>-</sup>, Myc, and Stat5b in pro–B cells in aged recipients to the levels observed in young recipients (Figure 7F; donor GFP<sup>+</sup> cells), providing a rationale for NRAS activation-dependent adaptation in the aged/inflammatory background. Notably, ERK activation in B progenitor cells was not affected by
The microenvironment in which the cells developed (Supplementary Figure 5A). AAT also prevented age-dependent increases in the expression of the inflammatory mediators TNF-α, IFN-α27, and MUC5B in B progenitors (Figure 7H and Supplementary Figure 5B). Note that we were unable to analyze NRASV12-initiated pro–B cells in the young or AATtg backgrounds, as these cells were not detectable above background levels (Figure 7D).

To corroborate these results using an alternative means to dampen age-dependent inflammation, we used IL-37tg mice. Similar to the above-described methods, young and old IL-37tg mice and age-matched controls were injected with the same pool of young BM progenitor cells transduced with retroviral vectors expressing NRASV12 plus GFP or CFP only. Expression of IL-37 in aged mice also prevented the selection of oncogenic NRASV12-expressing B progenitor cells (Figure 8A). Selection for NRASV12 was not observed in myeloid lineage fractions (Supplemental Figure 5C). Similar to findings in old AATtg recipients, young pro–B cell progenitors that developed in aged IL-37tg mice exhibited STAT5 activation and Hprt and Gmps gene expression levels similar to those detected in pro–B cells in young recipients, and NRASV12 expression prevented reductions in these fitness parameters in pro–B cells that developed in aged littermate control mice (Figure 8, B and C). In all, our experiments using both AATtg and IL-37tg models indicate that key fitness parameters of B cell progenitors can be dictated by the state of the microenvironment (age and inflammatory status) and that NRAS activation can rescue defects that result from aged/inflammatory microenvironments, facilitating clonal expansion of oncogenically initiated B progenitors.

Discussion

Aging is the single most important prognostic factor associated with the development of many diseases including cancer (1). The results described here demonstrate that aging-associated increases in inflammation negatively impact the development and function of B progenitor cells, including reductions in key indicators of cell fitness. Importantly, using two different, natu-
cytokines on B progenitor fitness in aged backgrounds, BCR-ABL and oncogenic NRAS\textsuperscript{V12} expression is associated with increased expression of inflammatory cytokines in old pro–B cells, perhaps further impairing the fitness of competing nononcogene-expressing progenitors (consistent with previous results in HSCs, refs. 55–57). We observed that either acute or chronic inflammation was sufficient to suppress key factors regulating normal B cell development (\textit{Pax5} and \textit{Ebf}) and function (\textit{Hprt} and \textit{Gmps}), resulting in significant decreases in B lymphopoiesis and pheno-
copying the aging process. From these studies, we conclude that inflammatory mediators negatively regulate B lymphopoiesis and accelerate reductions in B progenitor fitness, suggesting that chronic inflammation promotes B progenitor fitness impairments that manifest in old age.

Heterochronic parabiosis experiments in mice indicate that the aging process, for some organs and tissues, is substantially occurring molecules to reduce inflammation in old mice, we show that preventing aging-associated reductions in B progenitor fitness abrogates selection for NRAS\textsuperscript{V12}-initiated progenitors. These studies highlight how inflammation-induced alterations in the adaptive landscape in old age govern the selection of oncogenically initiated cells and, ultimately, leukemogenesis within hematopoietic progenitor cell populations (Figure 8D).

In recent years, there has been growing interest in identifying factors that regulate the aging process (8, 52, 53). We observed aging-associated increases in TNF-\(\alpha\), IL-6, and IL-1\(\beta\) levels in the BM of old mice. Previous studies demonstrate that aged BM stromal cells and mature B cells that accumulate in the BM are two sources of inflammation that manifest with age (25, 54). In this study, we further demonstrated that aged pro B cell progenitors also produce proinflammatory cytokines such as TNF-\(\alpha\). Notably, in addition to providing resistance to the effects of inflammatory cytokines on B progenitor fitness in aged backgrounds, BCR-ABL and oncogenic NRAS\textsuperscript{V12} expression is associated with increased expression of inflammatory cytokines in old pro–B cells, perhaps further impairing the fitness of competing nononcogene-expressing progenitors (consistent with previous results in HSCs, refs. 55–57). We observed that either acute or chronic inflammation was sufficient to suppress key factors regulating normal B cell development (\textit{Pax5} and \textit{Ebf}) and function (\textit{Hprt} and \textit{Gmps}), resulting in significant decreases in B lymphopoiesis and pheno-
copying the aging process. From these studies, we conclude that inflammatory mediators negatively regulate B lymphopoiesis and accelerate reductions in B progenitor fitness, suggesting that chronic inflammation promotes B progenitor fitness impairments that manifest in old age.
The study investigated the impact of aging and inflammation on hematopoiesis, focusing on the decline in B lymphopoiesis and the selection for oncogenic mutations. The authors observed a marked decline in B progenitor numbers in aged AATtg and IL-37tg mice. These results suggest that this decline in B lymphopoiesis may be an evolved program that reduces B cell production once a diverse B cell repertoire has developed during youth. The aging-associated biasing of hematopoiesis toward myelopoiesis may be advantageous in order to better combat infections and repair tissue injury. Nonetheless, both AAT and IL-37 expression prevented functional impairment in IL-7R signaling and the expression of purine synthesis genes in pro–B cell progenitors. Therefore, the late-life decline in the function of HSPCs (reduced fitness on a per-cell basis) can, in good measure, be explained by inflammation that increases late in life, beyond the period when reproduction is likely and thus largely outside the influence of natural selection.

In our mouse models, we observed selection for the tested oncogenic mutations within old B progenitor populations, leading to the development of B-lineage leukemias. This selection was not observed in young hematopoietic backgrounds due to the inability of oncogenic mutations to significantly improve the fitness of young B progenitor cells (thus, the highly fit B progenitors in young microenvironments provide effective competition influenced by non–cell-autonomous factors). Previous studies have shown that both cell autonomous and non–cell-autonomous (microenvironmental) factors contribute to hematopoietic aging, including functional changes in HSCs and B lymphopoiesis. Indeed, we showed that young BM progenitors transplanted into old mice (mildly conditioned with busulfan) produce B progenitors with signaling defects that mirror those of old progenitors, unless the old recipient mice express antiinflammatory mediators. Thus, the age and inflammatory status of the host mouse dictates the fitness of B progenitors produced by young HSCs in the host BM microenvironment. Nonetheless, our results also demonstrate that B lymphopoiesis derived from old HSCs transplanted into young mice still maintains aging-associated fitness defects, as indicated by substantial reductions in IL-7-mediated signaling and the expression of key metabolic and DNA replication genes. The inability of a young microenvironment to reverse aging-associated defects in hematopoiesis may result from substantial aging-associated genetic and epigenetic changes in HSPC populations, even when caused by cell-extrinsic factors such as inflammation.

Focusing on inflammation, we asked how age-dependent changes in the microenvironment could alter the fitness of B cell progenitors. Interestingly, we still observed a marked decline in B progenitor numbers in aged AATtg and IL-37tg mice. These results suggest that this decline in B lymphopoiesis may be an evolved program that reduces B cell production once a diverse B cell repertoire has developed during youth. The aging-associated biasing of hematopoiesis toward myelopoiesis may be advantageous in order to better combat infections and repair tissue injury. Nonetheless, we found that both AAT and IL-37 expression prevented functional impairment in IL-7R signaling and the expression of purine synthesis genes in pro–B cell progenitors. Therefore, the late-life decline in the function of HSPCs (reduced fitness on a per-cell basis) can, in good measure, be explained by inflammation that increases late in life, beyond the period when reproduction is likely and thus largely outside the influence of natural selection.

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Figure 8. Reducing inflammation in aged IL-37tg mice abrogates NRASV12-mediated oncogenesis. Young and old (2-month-old and 20-month-old, respectively) littermate (LC) or IL-37tg mice were transplanted with young NRASV12-expressing cells, as in Figure 7A. Three months after transplantation, BM was analyzed by flow cytometry for (A) the frequency of NRASV12-expressing cells in B progenitor cell populations and (B) activation of STAT5 and ERK in pro–B cells expressing NRASV12 (red-outlined gray boxes) or not expressing the oncogene. (C) mRNA levels of Hprt, Gmps, and Myc in sorted pro–B cells were determined by qPCR (note: NRASV12-initiated cells only expanded in the old littermate control mice). (D) Model for how aging and aging-associated inflammation regulate progenitor cell fitness and oncogenesis. Values in A–C represent the mean ± SEM, with more than 5 mice per group. *P < 0.05 and **P < 0.001, by Student’s t test relative to young controls.
to limit the expansion of oncogenically initiated progenitors. Notably, hematopoietic malignancies of the B lineage predomi-
nate in older mice, while in elderly humans, both B lymphoid 
and myeloid leukemias are common (63, 64). B lymphoid 
leukemias are also common in young children, and infections and the asso-
ciated inflammation have been proposed to play a role in their 
development (65). Whether in humans or mice, contexts that lead 
to reduced fitness of stem/progenitor cells would be expected to 
lead to selection for adaptive oncogenic events in these stem and 
progenitor cell populations and to malignancies of correspond-
ingly different types.

Inflammation has been associated with the progression of 
many solid tumors. Its actions in promoting tumorigenesis have 
largely been attributed to the ability of inflammatory cells to cre-
ate an immune-suppressive microenvironment, promote meta-
tasis, and enhance the protumorigenic phenotypes of cancer cells 
including proliferation, survival, and invasion (66, 67). Notably, 
our studies indicate that inflammation can promote selection 
for oncogenic events in HSPCs by suppressing overall progeni-
tor fitness, providing a context that favors oncogenic adaptation. 
These findings complement emerging studies that have shown 
increased hematopoietic clonal evolution (including for clones 
with known oncogenic drivers) in the elderly (68-72). Inflammation 
has also been proposed to contribute to the genesis of mye-
loproliferative disorders that are almost exclusively present in the 
erelderly (73, 74), and increased oncogenic clonal expansions and 
clonal hematopoiesis have also been demonstrated in old mice 
(75, 76). Notably, the incidence of leukemias is higher in individ-
uals with chronic inflammatory disease (77), although the cause 
of this association is unknown. Moreover, prophylactic aspirin 
use has been shown to reduce the incidence of colorectal cancer, 
breast cancer, and leukemias (78-80). Interestingly, polymor-
phisms associated with reduced inflammation are preferentially 
found in centenarians (81, 82).

Importantly, our studies provide a potential explanation for 
the mechanism underlying increased oncogenic clonal evolu-
tion and leukemias in old age. We demonstrated that the onco-
genes BCR-ABL, NRAS, and Myc can reverse aging-associated 
functional defects in B progenitors, promoting selection for 
progenitors expressing these oncogenes specifically in the aged 
hematopoietic context. These oncogenes do not restore differ-
entiation and likely convey other transformed phenotypes to the 
recipient cells. However, in the BM microenvironment of anti-
inflammatory aged mice, selection for NRASV12 was completely 
suppressed. We propose that the maintenance of hematopoietic 
progenitor fitness in both young and antiinflammatory, aged 
backgrounds can prevent oncogenic adaptation via stabilizing 
selection (35, 83). While oncogenic NRAS can restore fitness 
parameters (such as for signaling) in B progenitors (whether 
young or old) in an old hematopoietic background, it does not 
do so for progenitors in either young microenvironments or old 
but antiinflammatory microenvironments, as there appears to 
be little room for improvement.

In conclusion, our study demonstrates that aging is character-
ized by reductions in key parameters of B progenitor cell fitness 
and increased selection for oncogenic events, both of which are 
substantially modulated by the degree of aging-associated inflam-
mation. Overall our study indicates that dampening inflamma-
tion in older populations may reduce aging-associated functional 
impairment in hematopoiesis, which in turn may reduce leukemia 
incidence in these populations by preventing the selection for 
oncogenically initiated cells.

Methods
Microarray data were deposited in the NCBI’s Gene Expression 
Omni-
bus (GEO) database (GEO GSE67827). Microarray, mass spectrometry, 
and NMR methods are provided in the Supplemental Methods.

Mice
BALB/c and C57BL/6 mice of different ages were purchased from 
the National Institute of Aging (NIA) or the National Cancer Institute 
(NCI). CD45.1 congenic mice (B6.SJL-PtprcPepc/BoyCrCl) were purchased 
from Charles River Laboratories. TNF-αARE, AAT, and 
IL-3 transgenic mice were backcrossed onto a C57BL/6 background for 
more than 10 generations, and both transgene-positive and trans-
gene-negative littermates were aged in-house. Male and female mice 
from each strain were used in these studies.

Retroviral transduction and BM transplantation
Retroviral transductions and BM transplants were performed as 
previously described (84). The retroviral murine stem cell virus-
ires-GFP (MSCV-ires-GFP) (MiG), MiG-BCR-ABL, MiG-RAS, MiG-
Myc, and MSCV-ires-CFP (MiC) vectors were used to introduce the 
respective genes into MACS-purified (Miltenyi Biotec) murine c-KIT+ 
 hematopoietic progenitor cells. These cells were transduced with 
MiG viruses in nonadhesive 6-well plates by incubation at 37℃ for 
2 hours in the presence 8 μg/ml polybrene and 20 μg/ml stem cell fac-
tor, followed by the spinfection technique (centrifugation at 910 g for 
2 hours). Cells were then washed once with 1× PBS and transplanted 
into conditioned recipient mice.

Mice were condition using sublethal irradiation (5 Gy), lethal irra-
diation (10 Gy split dose), or busulfan (25 mg/kg) and injected with 
transduced cells 2–4 days after treatment (depending on the condi-
tioning protocol). In experiments in which recipient mice were lethally 
irradiated (Figure 2, G and H), mice were injected with 2 × 10^6 unstored 
BM cells from young (2-month-old) or aged (22- to 24-month-old) 
donor mice (prepopulated with young or aged competitors, respec-
tively). Before lethal irradiation, mice were deprived of food overnight 
to reduce small intestinal cytotoxicity. Four days after prepopulation, 
2 × 10^6 MiG-transduced c-KIT+ hematopoietic progenitor cells were 
then transplanted i.v. into recipient mice.

Inflammation-inducing and IL-7-neutralizing experiments
IL-7 neutralization experiments were performed as previously 
described (16). In these experiments, IL-7-neutralizing Abs were 
 injected at a dose of 0.5 mg/mouse every 4 days until the experiment 
was terminated. In the experiments described in Figure 4, LPS was 
injected at a dose of 1 mg/kg, and recombinant TNF-α was injected at a 
dose of 5 μg/mouse every 4 days until the experiment was terminated.

EdU analysis of B progenitor populations
EdU analysis of B cell progenitor populations was performed using the 
Click-IT EdU Alexa Fluor 647 Imaging Kit (Life Technologies; catalog 
C10634). C57BL/6 mice were injected i.p. with EdU (2 mg/mouse,
dissolved in PBS) for 2 hours. After 2 hours, mice were sacrificed, and 10^6 BM cells were collected and surface stained to identify pro–, pre–, and immature B cell progenitor populations. After staining, cells were washed once with 1× PBS and the pellets resuspended in 100 μl Click-iT fixative (Life Technologies) for 15 minutes at room temperature. After fixation, cells were again washed and incubated for 10 minutes in 50 μl diluted Click-iT saponin-based permeabilization solution (Life Technologies). After the 10-minute incubation period, 250 μl reaction mix (including 1× PBS, CuSO_4, Alexa Fluor 647, and reaction buffer additive, combined following the manufacturer’s protocol) was added to each sample and incubated for 30 minutes at room temperature. Next, cells were washed and resuspended in 1× PBS containing 50 pg/ml PI (Roche). Samples were run on a Galileo cytometer (Beckman Coulter). The normalized x-mean mean fluorescence intensity (MFI) of EdU^+ cell populations (which is a measure of S-phase progression) was calculated using the following formula: [(x-mean MFI of EdU^+ cells) – (x-mean MFI of the G_1 population)]

Flow cytometric analysis

Surface staining. Single-cell suspensions were plated in 96-well round-bottomed plates and washed in FACS buffer containing 3% FBS, 1× PBS, and 2 mM EDTA (vol/vol). After washing, cells were surface stained for 1 hour on ice to identify the hematopoietic cell population of interest (cells were stained in 50 μl Ab solution). Cells were washed once with 200 μl FACS buffer and resuspended in 400 μl FACS S buffer for flow cytometric analysis.

The following anti-mouse Abs were used: phycoerythrin-conjugated (PE-conjugated) anti-B220 (BD Pharmingen; catalog 553090), anti-CD43 (BD Pharmingen; catalog 553271), anti-CD4 (eBioscience; catalog 12-0041-82), anti-CD8 (eBioscience; catalog 12-0081-82), anti-Ter119 (eBioscience; catalog 12-5921-81), anti-CD48 (BD Pharmingen; catalog 557485), anti-MAc1 (BD Pharmingen; catalog 553979), and anti-CD45.2 (BD Pharmingen; catalog 560695); PE-Cy7-linked anti-MAc1 (eBioscience; catalog 25-0112-82), anti-IL-7Rα (eBioscience; catalog 25-1271-82), anti-CD45.1 (eBioscience; catalog 25-0453-82), and streptavidin (eBioscience; catalog 25-4317-82); allophtocyanin-linked anti-B220 (eBioscience; catalog 17-0452-82), anti-Sca1 (eBioscience; catalog 17-5981-82), and anti-CD150 (eBioscience; catalog 17-1502-80); Pacific Blue–linked streptavidin (e450) (eBioscience; catalog 48-4317-82) and anti-CD93 (eBioscience; catalog 48-5892-80); FITC-conjugated anti-c–KIT (BD Pharmingen; catalog 553354); and biotin-linked anti-CD93 (eBioscience; catalog 13-5892-82). Flow cytometric analysis was performed on a CyAn, Cytomics FC 500, or Cell Lab Quanta SC flow cytometer (all from Beckman Coulter). B cell progenitors were defined on the basis of their expression of surface markers: pro–B cells: B220^lo, CD93^lo, CD43^lo/med; pre–B cells: B220^hi, CD93^hi, CD43^hi; immature B cells: B220^lo, CD93^lo, CD43^med/hi; mature B cells: B220^hi, CD93^hi, CD43^hi. Cell sorting was performed on MoFlo XDP 70 and 100 cell sorters (Beckman Coulter).

Intracellular staining. Intracellular cytokine staining was performed as previously described (16). The following BD Biosciences PE-conjugated Abs were used: p-STAT3 (P-705, catalog 612569), p-STAT5 (P-Y694, catalog 612567), p-ERK1/2 (P-T202/P-Y204, catalog 612566), and control (rat IgG2b, catalog 556925). Anti-TNF-α PE-conjugated Ab (BioLegend, catalog 506305) was used for the detection of TNF-α. Stained cells were analyzed on the CyAn ADP Analyzer (Beckman Coulter).

Statistics

Unpaired t tests, Cox proportional hazards tests, and 1-way ANOVA were used to analyze the data, with a P value of less than 0.05 considered statistically significant. All error bars represent biological replicates (different mice), not technical replicates. Statistical analyses were performed with GraphPad Prism software, version 6.07 (GraphPad Software). All results are expressed as the mean ± SEM.

Study approval

All animal experiments were approved by and performed in accordance with guidelines of the IACUC of the University of Colorado AMC.

Author contributions

CJH contributed to the study design, collected and interpreted data, created the manuscript figures, provided financial support for the study, and drafted and revised the manuscript. MCS assisted with the experiments depicted in Figure 1, D–G. J Kim, LA, and ACT provided bioinformatic assistance with the microarray data. VZ performed in vivo experiments and managed the mouse colonies. AED contributed to experiments shown in Figure 2, A and B. LJ performed the experiments presented in Supplemental Figure 1B. TA performed IL-1β ELISAs. ENM and ETC provided the TNF-αARE transgenic mice and proposed study designs. JKlaction and NJS assisted CJH with mass spectrometry and the untargeted quantitative 1H NMR metabolomics experiments, respectively. CAD contributed the AATtg and IL-37tg mice, proposed study designs, and reviewed the manuscript. JD conceived the study, provided financial support for the study, analyzed data, and assisted with the drafting of the manuscript. All authors read and approved the final manuscript.

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