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An aging mouse model of human chronic myeloid leukemia

Taisen Hao, PhD[1], **Chunxiao Zhang, PhD**[1], **Zhiqiang Wang, PhD**[1], **Alison Buck**[2], **Steven L. Vonderfecht, DVM, PhD**[3], **Richard Ermel, DVM, PhD**[4], **Young Kim, MD**[5], **WenYong Chen, PhD**[1,6]

[1]Department of Cancer Biology, The Beckman Research Institute of City of Hope, Duarte, CA 91010.

[2]Eugene and Ruth Roberts Summer Student Academy of City of Hope, Duarte, CA 91010

[3]Consultant in Veterinary Pathology, Reno, NV 89521

[4]Center for Comparative Medicine, The Beckman Research Institute of City of Hope, Duarte, CA 91010.

[5]Department of Pathology, City of Hope National Medical Center, Duarte, CA 91010.

Abstract

Chronic myeloid leukemia (CML) is an age-dependent blood malignancy. Like many other age-dependent human diseases, laboratory animal research of CML uses young mice that do not factor in the influence of aging. To understand how aging may impact animal modeling of human age-dependent diseases, we established the first aging mouse model of human CML in BALB/c mice in the advanced age defined by 75% survival. This model was developed by noncytotoxic depletion of bone marrow lineage-positive cells followed by BCR-ABL retroviral transduction and transplantation. CML developed in aging mice shared many similarities to that in young mice, but had increased incidence of anemia that is often seen in human CML. Importantly, we showed that aging of both donor hematopoietic stem cells and recipient bone marrow niche impacted BCR-ABL mediated leukemogenesis and leukemia spectrum. Optimal CML induction relied on age-matching for donors and recipients, and cross-transplantation between young and old mice produced a mixture of different leukemia. Therefore, our model provides initial evidence of the feasibility and merit of CML modeling in aging mice and offers a new tool for future studies of CML stem cell drug resistance and therapeutic intervention in which aging would be taken into consideration as an influencing factor.

[6]Correspondence should be addressed to: WenYong Chen, Ph.D., Department of Cancer Biology, Research, Beckman Research Institute, City of Hope, Duarte, CA 91010, Tel: 626-218-8911; Fax: 626-218-7193, wechen@coh.org.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

INTRODUCTION

Most human diseases are age-dependent, but the majority of their research in laboratory animals has been done on young animals. It is unclear whether older animals may be more appropriate for study of human diseases that are prevalent in older humans. In a trans-NIH (National Institutes of Health) initiative to determine whether the age of the model organism influences experimental outcomes and whether older animals are better models of experimental studies for conditions, interventions, diseases or exposures for which aging is a risk factor of the human condition, we set out to establish an aging mouse model of human chronic myeloid leukemia (CML) that is a classic age-dependent lethal blood malignancy with a median age of diagnosis around 60 (ref 1,2). CML has been a model disease for cancer research for decades, which has resulted in a series of breakthrough in our understanding of cancer and inventing treatments.

CML, if untreated, progresses slowly from chronic phase to accelerated and then blastic phases that are characterized by the increasing percentage of blast cells in the circulation[1]. Historically, cytogenetic studies led by Nowell and Hungerford in the 1960s identified a consistent and characteristic small chromosome abnormality within the neoplastic cells of patients with CML, which was later named as the Philadelphia chromosome.[3] In the 1970s to 1980s, the Philadelphia chromosome was further confirmed to arise from a reciprocal translocation between two genes, Abelson murine leukemia viral oncogene homolog (ABL) on chromosome 9 and breakpoint cluster region (BCR) on chromosome 22 (ref 4–6). In the 1990s, the hybrid protein product of the BCR-ABL fusion gene encoding an aberrant tyrosine kinase was demonstrated to be sufficient to drive CML development.[7–10]

Hematopoietic stem cells (HSCs) have been identified as the cell of origin for CML.[11,12] BCR-ABL transforms HSCs into leukemic stem cells (LSCs) that are responsible for driving disease initiation, progression, and potential relapse of leukemia therapy.[13] LSCs share a similar capacity to HSCs of self-renewal and generating various progenitor cells; however, LSCs attain altered immune-phenotypes, enhanced cellular survival, as well as uncontrolled proliferation.[14] Imatinib mesylate, a tyrosine kinase inhibitor (TKI), was the first successful targeted therapy in human cancer.[15] It was approved by the U.S. FDA as the first-line treatment for Philadelphia chromosome-positive CML in 2001. Newer TKIs including nilotinib, dasatinib, bosutinib, and ponatinib have been developed to supplement imatinib in clinics with higher potency and better molecular responses.[16] Even though the mortality rate has reduced dramatically since the introduction of TKIs.[17] these drugs don’t eradicate CML LSCs in most patients and the disease relapses after drug discontinuation.[18–21] The resistant LSC subpopulation serves as a reservoir to reinitiate leukemia post-TKIs. Therefore, eliminating LSCs is considered a crucial step to achieve a real cure for CML patients. Gaining insights into the mechanisms of LSCs drug resistance and novel therapeutic strategies with physiologically relevant CML models is critical.

Extensive efforts have been devoted to developing CML mouse models since the early 1990s. Several groups demonstrated a pivotal role of BCR-ABL in transformation and leukemogenesis in mice by retroviral transduction of murine bone marrow cells followed by bone marrow transplantation (BMT) into lethally irradiated recipients, in which BALB/c

mice were used mostly. In these studies, 210 KD BCR-ABL (p210), the main fusion type in human CML produced CML-like myeloproliferative disease in mice (mouse CML hereafter). However, CML was produced nonexclusively in these models along with other types of leukemia that possibly resulted from the viral transformation of committed progenitors other than HSCs.[7–10] Even though these early exploratory studies provided insights into the generation of CML mouse models, the application of these methods was severely limited by a low frequency of CML occurrence. In order to overcome these limitations, several groups refined the BALB/c mouse model of CML by incorporating a myeloablative reagent (5-fluorouracil, 5-FU) for priming donor bone marrow to reduce committed progenitors and lineage cells. This method successfully generated exclusive CML phenotypes resembling human chronic CML with 100% disease penetrance and high reproducibility.[22–24] The BALB/c CML model has since become a widely used model for studying CML disease progression, mechanisms, and therapeutic interventions, which contributes significantly to our understanding of this disease.[25–33] However, in contrast to human CML that occurs mostly in the elderly, CML mouse models are generated in very young mice (2 to 3 months). It is unclear how advanced age and aging microenvironment may influence leukemogenesis, therapeutic response and LSC persistence in the CML mouse models. The same situation is true for other leukemia and solid tumor studies. High cost for maintenance of aging mice and long turnover time are two major practical factors limiting the use of old mice for disease modeling. Scientifically, it is unclear whether modeling human diseases in old animals is feasible and meritful. In this study, we explored the feasibility and potential of utilizing BALB/c mice in advanced age as a model system for human CML.

RESULTS

Aging of BALB/c mice and terminal blood differentiation changes

The median lifespan for BALB/c mice is 757 days (about 25 months) for females and 707 days for males (about 23 months), and thus 24 months on average for both sexes.[34] The advanced (or old) age was defined here as age greater than the “75% survival” level observed for the strain. For BALB/c mice, the calculated age for 75% survival is about 18 months. During the aging process, we observed similar conditions previously reported for aging BALB/c mice.[35] The premature euthanization occurred mostly due to non-neoplastic conditions including fighting wounds and ulceration that occurred more frequently in males, possibly due to group-caging.

To determine the impact of chronological aging on hematology in BALB/c mice, we analyzed baseline hematological parameters of “grossly normal” mice (see exclusion and inclusion criteria in the Methods) at the age of 3, 6, 12 and 18 months. Peripheral blood analyzed from mice of both sexes showed that a progressively significant increase of neutrophil (NE) count from 3-month to 18-month, but not lymphocytes (LY). Total white blood cell (WBC) count increased from 3-month to 12-month, but no statistically significant change was observed at 18 months, possibly due to a slight drop of LY count albeit a significant increase of NE count in the blood (Figure 1A). Red blood cell (RBC) count showed no significant change across the age points sampled (Figure 1B). Percentagewise,

NE (%) significantly increased and LY (%) significantly decreased after 12 months (Figure 1C and D), but most mice retained more than 50% LY at 18 months. These results suggest a moderately biased differentiation towards myeloid lineages by 18 months of age in BALB/c mice.

5-FU myeloablation protocol was not suitable for inducing CML in old mice

Previous studies established an efficient modeling strategy to induce CML-like disease in young mice. This strategy utilizes 5-FU as a priming reagent for donor bone marrow to enrich HSCs for transduction and BMT (Figure 2A), and CML is quickly induced within 5 weeks.[22] Rapid induction of CML is also desired in old mice so that complication of age-related spontaneous tumors and deaths would be minimized. We first used this protocol to induce CML in “grossly normal” mice in the advanced age (16 to 20 months, and 18 months on average). We titrated the lethal dose of γ-radiation in old BALB/c mice. The minimal dose to induce 100% lethality in two weeks was 800 rads (Supplemental Figure 1). To achieve more consistent results, 900 rads were used for the studies, which was the same as in young BALB/c mice. We primed 18-month-old donor mice with 5-FU prior to bone marrow harvest, and retroviral transduction with BCR-ABL vector MIG210 bearing a GFP-expression cassette was carried out as we described previously.[27] Increasing dosages of transduced cells from 0.1 to 0.6 million (m)/mouse were transplanted into lethally irradiated 18-month recipient mice. Surprisingly, we found that 0.1m cells failed to engraft recipient mice, and 0.2m cells engrafted mice but failed to develop CML in old mice over 70 days (Figure 2B, C). Only 2 out of 4 mice in 0.6m group developed CML, but 1 out of 4 mice in 0.4m group developed B-cell acute lymphoid leukemia (B-ALL) in the same period with massive number of $B220^+$ blasts (Figure 2B, C and not shown). In contrast, MIG210 transduction in young mice as a positive control developed CML rapidly, and all mice succumbed to CML-mediated death by 40 days (Figure 2C), which is consistent with our previous report.[27] None of the old mice with MIGR1 empty vector transduction produced CML (Figure 2C). All old mice receiving 0.2m and 0.4m MIG210 transduced cells eventually died after 5 to 6 months (Supplemental Figure 2). However, such prolonged disease latency would not be desired because the results could be confounded by the increased chance of spontaneous malignancies and age-related death towards the end of life. We observed a typical transduction efficiency (20–30% GFP^+) with the 5-FU protocol, and further increasing transduction efficiency by culturing cells in retronectin-coated plates did not improve CML development (not shown). The above results were unexpected given that 0.1m and 0.2m young donor cells are sufficient to confer 100% CML development in young BALB/c recipients in 40 to 60 days using the 5-FU protocol.[27]

5-FU is a thymidylate synthase inhibitor that blocks DNA replication and causes death in dividing cells.[36] 5-FU priming of bone marrow kills proliferating progenitor and precursor cells to inhibit development of other leukemia such as B-ALL, and simultaneously enriches non-dividing young HSCs for BCR-ABL transformation and CML development.[22] However, aging HSCs expand the population with more cell cycle entry and accumulating cellular damages.[37] We hypothesized that aging stem/progenitor cells might become more susceptible to the cytotoxicity of 5-FU, which may contribute to the inefficient bone marrow engraftment and BCR-ABL transformation in old mice. We analyzed the apoptotic rate of

lineage negative (Lin^-) bone marrow cells collected from donor mice treated with 5-FU or vehicle. Compared to the 3-month group, Lin^- cells in the 18-month group showed a much higher apoptotic rate when treated with 5-FU versus the vehicle (Figure 2D), indicating that aging stem/progenitor cells were more sensitive to 5-FU treatment compared to their young counterparts. In addition, BCR-ABL expression increases reactive oxygen species that cause cellular DNA damage,[38] which may further aggravate the cytotoxicity of 5-FU at the early stage of HSC transformation in old mice. Therefore, a less invasive protocol needs to be adopted in order to successfully model CML in aging mice.

A new and efficient CML induction protocol in aging mice by noncytotoxic lineage depletion

To overcome the drawback of 5-FU for aging mice, we opted to use immunomagnetic beads to deplete lineage positive (Lin^+) bone marrow cells, which typically yields an enrichment of Lin^- cells in 90% of the pass-through (unlabeled) cell fraction. We hypothesized this would mimic the effect of 5-FU to reduce committed progenitors or precursors while enrich HSCs without exposing them to a cytotoxic agent. Previous studies also showed that efficient CML induction depends on efficient retroviral transduction.[22–24] Towards this goal, we utilized the recombinant human fibronectin fragment (Retronectin) that promotes the colocalization of retroviral particles and target cells to boost the transduction ratio. As outlined in Figure 3A, the unlabeled Lin^- cells after immunomagnetic depletion of Lin^+ cells were used for two rounds of MIG210 transduction in Retronectin-coated plates. With greatly reduced cell number for the transduction, higher multiplicity of infection (2 for each round in this study) was accomplished easily. Importantly, the viability rate of aging bone marrow cells after two rounds of MIG210 transduction was substantially improved to 70 to 90% as compared to their poor viability (30 to 40%) with the 5-FU protocol.

Using this lineage depletion protocol, we found that transplantation with three dosages (0.1, 0.2, and 0.4 million) of the transduced cells showed that aging mice developed exclusively CML phenotypes with 100% disease penetrance. Survival data demonstrated that all aging CML mice died in 38 to 63 days (Figure 3B), on par with the 5-FU protocol in young mice regarding the kinetics of disease progression. Elevated WBC and NE counts from peripheral blood were observed in 0.2m and 0.4m groups starting at 3 weeks post-BMT (Figure 3C), and higher values of these counts were observed at 4 weeks post-BMT in 0.2m group than in 0.1m group (Figure 3D). Immunophenotyping of the CML cells in blood confirmed that most WBCs were GFP^+ and nearly all GFP^+ cells belonged to $Mac1/Gr1^+$ myeloid lineage (Figure 3E). Notably, mild to severe anemia was observed in a portion of the CML mice (Figure 3F, compared to normal mice in Figure 1B). Furthermore, we found that $GFP^+Lin^-CD150^-$, but not $GFP^+Lin^-CD150^+$, CML cells in aging mice passaged the disease to secondary transplants (Figure 3G). This is consistent with our previous finding in the young BALB/c mouse model that CML LSCs reside in the Lin^-CD150^- cell fraction.[39] With the new protocol, we found that young and old mouse Lin^- bone marrow cells were transduced by MIG210 with similar efficiency (Supplemental Figure 3), and CML was successfully produced in all young mice as described further below.

CML in aging mice displayed similar histopathological features as in young mice,[22,27] including the overcrowding of mature neutrophils in the peripheral blood, splenomegaly with massive infiltration of myeloid cells in various stages of differentiation and with high mitotic index, distention and hypercellularity of hepatic sinusoids due to accumulation of clusters of immature myeloid and erythroid cells, and pulmonary involvement characterized by hypercellularity and thickening of alveolar interstitium due to accumulation of cells thought to be immature myeloid cells within alveolar capillaries (Figure 4A). Noticeably, clusters of immature erythroid cells were present in spleen and liver alongside CML cells (Figure 4A,B). Histiocytic sarcoma was frequently found within spleen and liver of aging CML mice (Figure 4B), a phenomenon that is also often observed in CML of young mice.[40] Histiocytic sarcoma can be derived from the same BCR-ABL transduced stem/progenitor cells that produce excessive neutrophils in young CML mice,[40] but can also be a spontaneous neoplasm unrelated to BCR-ABL induction especially in old BALB/c mice.[35] Mice with primary histiocytic sarcoma of spontaneous nature could have increased neutrophils in blood; however, the majority of WBCs of aging CML mice were GFP^+ neutrophils, suggesting that CML but not histiocytic sarcoma was the primary contributor for myeloproliferation in these mice. Aided by the exclusion criteria of aging mice for the study, we did not detect other large spontaneous solid tumors associated with these CML mice at necropsy, confirming that CML was their main cause of death. Together, our results support that it is feasible to produce CML in BALB/c mice in advanced age with the non-cytotoxic lineage-depletion protocol, and that CML in aging mice shares many similarities with CML in young mice and is not confounded by other age-related tumors.

Impact of HSC and bone marrow niche aging on BCR-ABL transformation and leukemogenesis

Aging impacts HSC function on the molecular, cellular and extracellular levels. Aging HSCs undergo intrinsic genetic, epigenetic and metabolic changes resulting in alterations in HSC regeneration and differentiation.[41] Aging also changes bone marrow microenvironment especially for niche composition and chemokine/cytokine production that affect the function of HSCs.[42] However, how aging-mediated intrinsic HSC changes and extrinsic bone marrow niche changes may mutually influence oncogenic transformation of HSCs and leukemia development is poorly understood. Using our new mouse model, we examined the impact of HSC and niche aging on the BCR-ABL transformation of HSCs and CML development. We transduced young and old BALB/c donor cells with MIG210 side-by-side under the same conditions, and each group of donor cells were transplanted to both young and old recipients side-by-side (Figure 5A). The impact of HSC aging would be determined by comparing young vs old donor cells to the same recipients, and the impact of niche aging determined by comparing the same donor cells to young vs old recipients.

Both young donor to young recipient (Y-Y) and old donor to old recipient (O-O) BMT groups generated exclusively CML with similar kinetics and pathological phenotypes. However, longer disease latency and mixed leukemia phenotypes were observed in both young to old (Y-O) and old to young (O-Y) BMT groups (Figure 5B–F). Peripheral blood sampled at week 4 post-BMT presented significantly elevated WBC and NE counts in both the Y-Y and O-O but not in Y-O or O-Y groups (Figure 5C), but the increased NE

percentage was seen in all four groups (Figure 5D, compared to normal mice in Figure 1C, D). This indicates that all groups underwent myeloid-biased differentiation, with faster disease progression in Y-Y and O-O groups that was in line with their shorter survival in these two groups. Notably, RBC count in the O-O group was significantly lower than that in the Y-Y group (Figure 5E), suggesting mild anemia in O-O mice as described above. Immunophenotyping of blood nucleated cells from the diseased mice showed that over 95% of the GFP^+ fractions in the Y-Y and O-O groups were $Mac1/Gr1^+$ myeloid cells (Figure 5F). In contrast, only two mice each in the O-Y and Y-O groups showed CML phenotypes similar to that of the Y-Y and O-O groups. For the rest of the four mice in the O-Y group, the GFP^+ fractions were mainly lineage negative cells that were stained by neither Mac1/Gr1 nor B220, indicating possibly undifferentiated leukemia. For the Y-O group, one mouse developed mixed phenotype acute leukemia that contained about 10% of Mac1/Gr1 and B220 double-positive cells along with 88% of $Mac1/Gr1^+$ cells, while three other mice showed similar undifferentiated leukemia phenotype as in the O-Y group (Figure 5F). The blood smear sampled from the diseased mice further confirmed that Y-Y and O-O BMT generated exclusively CML phenotypes with overpopulating mature neutrophils in the blood; in contrast, numerous immature cells or blasts were observed from O-Y and Y-O groups (Figure 5G).

For comparing the impact of HSC aging, young HSCs were more efficiently transformed for chronic CML development than old HSCs when the recipients were young (Y-Y vs O-Y), but old HSCs were more efficiently transformed for CML when the recipients were old (Y-O vs O-O). For comparing the impact of niche aging, young bone marrow niche better supported CML transformation and disease progression when the donors were young (Y-Y vs Y-O), but old niche better supported CML development when the donors were old (O-Y vs O-O). Therefore, both HSC aging and niche aging impacted BCR-ABL transformation of HSCs and leukemogenesis, with young HSCs favoring young niche for BCR-ABL transformation and chronic CML development while old HSCs favoring old niche. Unmatching the donor and recipient age slowed the initiation of leukemogenesis but led to a major shift in leukemia spectrum from chronic CML to predominantly less differentiated leukemia. These results suggest that interaction of BCR-ABL transformed HSCs with bone marrow niche likely plays a crucial role in determining leukemia subtypes or spectrum.

DISCUSSION

In this study, we have successfully established the first aging mouse model of human CML using an efficient novel CML induction protocol by immunomagnetic depletion of lineage-positive cells followed by BCR-ABL retroviral transduction and transplantation. This protocol overcomes the drawback of cytotoxic effect of 5-FU for priming donor mouse bone marrow for CML induction in aging mice that caused massive cell death both *in vivo* and *in vitro*. Immunomagnetic depletion of lineage-positive cells has achieved similar effects to 5-FU for enriching HSCs and reducing committed progenitors and precursor cells, but without toxicity to cells, which helps CML induction with 100% penetrance in aging mice. This new protocol also works for CML induction in young mice.

CML in aging mice exhibited many pathological similarities to that in young mice previously characterized. However, CML in aging mice were more prone to develop anemia than in young mice. It was noted that infiltrates of CML in spleen and liver from old mice were often accompanied by clusters of immature erythroid cells. Intriguingly, anemia is also common in human chronic CML, in about 30% of the cases. Anemia in human CML is associated with lower quality of life and higher baseline risks; however, anemia may or may not be linked to poorer response to tyrosine kinase inhibitors and reduced overall survival, depending on the studies.[43–46] Some human CML patients receiving HSC transplantation or interferon therapy also develop anemia.[47] Although the underlying causes for anemia in CML patients and aging CML mice are not clear, it may be related to age, radiation, transplantation, therapy, and other age-related changes of bone marrow.[44,47] In this regard, aging mice may model human CML pathogenesis better than young mice.

Bone marrow microenvironment plays an important role in mediating leukemogenesis and leukemia cell drug response, but such a role of bone marrow niche is likely dependent on leukemia subtypes.[48] BCR-ABL is a cause for chronic CML, and the disease can progress to acute myeloid or lymphoblastic leukemia at the late stage. Many genetic and epigenetic alterations have been proposed to drive blastic transformation of CML,[49] but the mechanisms of such transformation remains poorly understood. In addition, BCR-ABL is also a cause for a subtype of mixed phenotype acute leukemia (MPAL) in humans that display features of both acute myeloid leukemia and acute lymphoblastic leukemia.[50] Recently, HSCs have been identified as the cell of origin for MPAL.[51] Therefore, BCR-ABL is able to transform HSCs into different types of leukemia. Our cross-age transplantation studies indicate that BCR-ABL mediated leukemogenesis is possibly determined by proper interaction of BCR-ABL transformed HSCs with bone marrow niche, in that age-matched donors and recipients are preferred for efficient CML development while unmatching donor and recipient age alters the disease course that favors less differentiated leukemia and MPAL development. However, the underlying mechanisms remain to be discovered. We speculate that the mismatched signaling interplay between newly transformed HSCs and the microenvironmental stimulus could contribute to this phenomenon. It is known that certain cellular phenotypes may change drastically with aging, including the expression of cell surface markers that are sensitive to environmental cytokines and chemokines[52,53]. Notably, expression of a small RhoGTPase Cdc42 that regulates cell-cell and cell-extracellular matrix adhesion is increased significantly in aged HSCs causing a loss of cellular polarity and functional defects.[54] On the other hand, stromal support network goes through significant changes with aging as well. Aged bone marrow microenvironment presents a transcriptional profile that is very similar to an infection-induced bone marrow stroma.[55] With cross-age transplantation, the transformed HSCs might not recognize or respond to the mismatched bone marrow microenvironmental stimulus, resulting in obstructed differentiation and abnormal phenotypes. Further transcriptional and phenotypic profiling could be utilized to uncover the delicate nature of this phenomenon in the future. Nonetheless, our results provide novel insight into BCR-ABL leukemogenesis, and it is tempting to speculate that the changes in bone marrow microenvironment may contribute to blastic transformation of human chronic CML.

Our new aging mouse model of CML confers an innovative tool for future studies that would consider aging as an influencing factor. This may be meritful for addressing age-related issues, such as the impact of bone marrow microenvironment aging on leukemogenesis and LSCs, HSC aging on LSC functions and drug resistance, aging influence on anti-leukemia drug response and effectiveness, etc. However, given the high cost for aging mouse colonies and that many features of CML in aging mice are similar to those in young mice, one should consider the cost and effectiveness for using the aging vs young mouse model of CML in his/her studies.

MATERIALS AND METHODS

Aging BALB/c mouse colony

The use of animals was approved by the City of Hope Institutional Animal Care and Use Committee. The initial BALB/c breeder mice were purchased from Taconic. Mice were housed in specific-pathogen-free facilities with an ambient temperature at 20 to 22°C and 12-hour circadian light/dark cycle. Male and female mice were separately group-caged with up to 5 mice/cage using an individually ventilated caging system. If fighting occurred inside the cages, the aggressive mice (mostly males) were removed for individual caging. Air ventilation rates were routinely maintained to ensure consistent air flow among racks and cages. All cages were provided with sterile Sani-chips beddings as well as plastic toys to improve mouse social activities. Each room was assigned with a designated animal care technician for daily cage and room maintenance, and animal health was routinely checked by animal health technicians and veterinarians. Mice used for the study were non-breeders and were fed ad libitum with PicoLab Diet 5053 for the aging process until bone marrow transplantation.

Inclusion and exclusion criteria of aging BALB/c mice for the study

Several non-neoplastic and neoplastic conditions may affect aging BALB/c mice. The common non-neoplastic conditions include spontaneous corneal opacities, ulcerative blepharitis and periorbital abscesses, hearing loss, and age-related cardiopathy or cardiomyopathy[35] along with fighting wounds and tissue damages. The common neoplastic conditions in the old BALB/c mice include reticular neoplasia (histiocytic sarcoma and lymphoma), lung tumors, Harderian gland tumors, adrenocortical adenomas, liver tumors and mammary adenocarcinomas. Other rare tumors in old BALB/c mice include hemangiosarcomas, ovarian tumors, and Leydig cell tumors of the testicle.[35] Aging mice with visible or palpable tumors, open wounds, severe ulceration and inflammation, abnormal breathing, and unusual body postures were excluded from the study and euthanized according to the established institutional criteria. Mice included in the study were grossly normal, but could have conditions like corneal opacities, hearing loss, or blepharitis and abscesses under control that don’t interfere leukemia development.

Bone marrow transduction and transplantation

The conventional protocol for bone marrow transduction and transplantation was conducted as described previously,[27,39] in which mouse bone marrow was harvested three days after 5-FU priming, and nucleated cells were transduced by ecotropic BCR-ABL vector MIG210 or

control vector MIGR1 that were packaged using Phoenix-Eco cells to produce viral stocks with titers ≥2×10^6/ml for transduction.[27] To transduce aging mouse bone marrow cells, we used a modified protocol. Aging donor bone marrow was harvested without 5-FU priming, and total nucleated cells were collected by lysing red blood cells with ACK lysing buffer (Gibco). Hematopoietic stem/progenitors cells were enriched by depleting lineage-positive cells using EasySep mouse hematopoietic progenitor cell isolation kit (StemCell Technologies) according to the manual instruction, and then cultured in stimulation medium (DMEM, 10% heat-inactivated FBS, 1% penicillin/streptomycin, 120 U/ml of recombinant murine IL-3, 500 U/ml of IL-6, and 5 U/ml of SCF [Peprotech]) at 1 million cells per milliliter for 24 hours prior to retroviral transduction. Two rounds of cosedimentation retroviral transduction were performed as described,[27] but in 24-well plates coated with Retronectin [15 μg/well (Takara Bio)], at multiplicity of infection of 2 for each round. Three days before lethal irradiation, recipient mice were placed under Sulfatrim antibiotic diet and remained on this diet for at least three months of the study. Old recipient mice were lethally irradiated by 900 Rads (as in young mice) that was split into 2 doses (2 × 450) and separated by 3 hours. Mice were then randomly distributed among study groups. Both sexes were included and when possible, evenly distributed among the groups. The group allocation was not blinded to the investigators. The group size was determined as described[56] and adjusted according to our previous experience with CML mice especially with low numbers in dose titration assays.[39] The transduced cells were transplanted through retro-orbital injection. The tranusduction and transplantation experiment had been replicated multiple times in the lab.

Blood analysis and mouse hematopoietic stem/progenitor cell isolation

Mouse peripheral blood cell counts were analyzed using a five-part differential hematology analyzer HemaVet. BD Pharmingen PE-labeled lineage antibodies (Gr-1, clone RB6–8C5; CD11b/Mac-1, clone M 1/70; B220, clone RA3–6B2; Ter119, clone TER-199; and CD3e, clone 145–2C11) were used for lineage detection by flow cytometry. Data were collected on a five-laser BD-LSR-Fortessa flow cytometer and analyzed with Flowjo software.

Statistical analysis

For animal survival curves, Kaplan-Meier survival analysis was performed, and the statistical significance was determined with the log-rank test. For other statistical analysis, either the student t-test or ordinary one-way ANOVA analysis was performed where appropriate. The two-tailed analysis was used in all studies with significance value (p) less than 0.05 was considered statistically significant. Error bars were shown as s.d.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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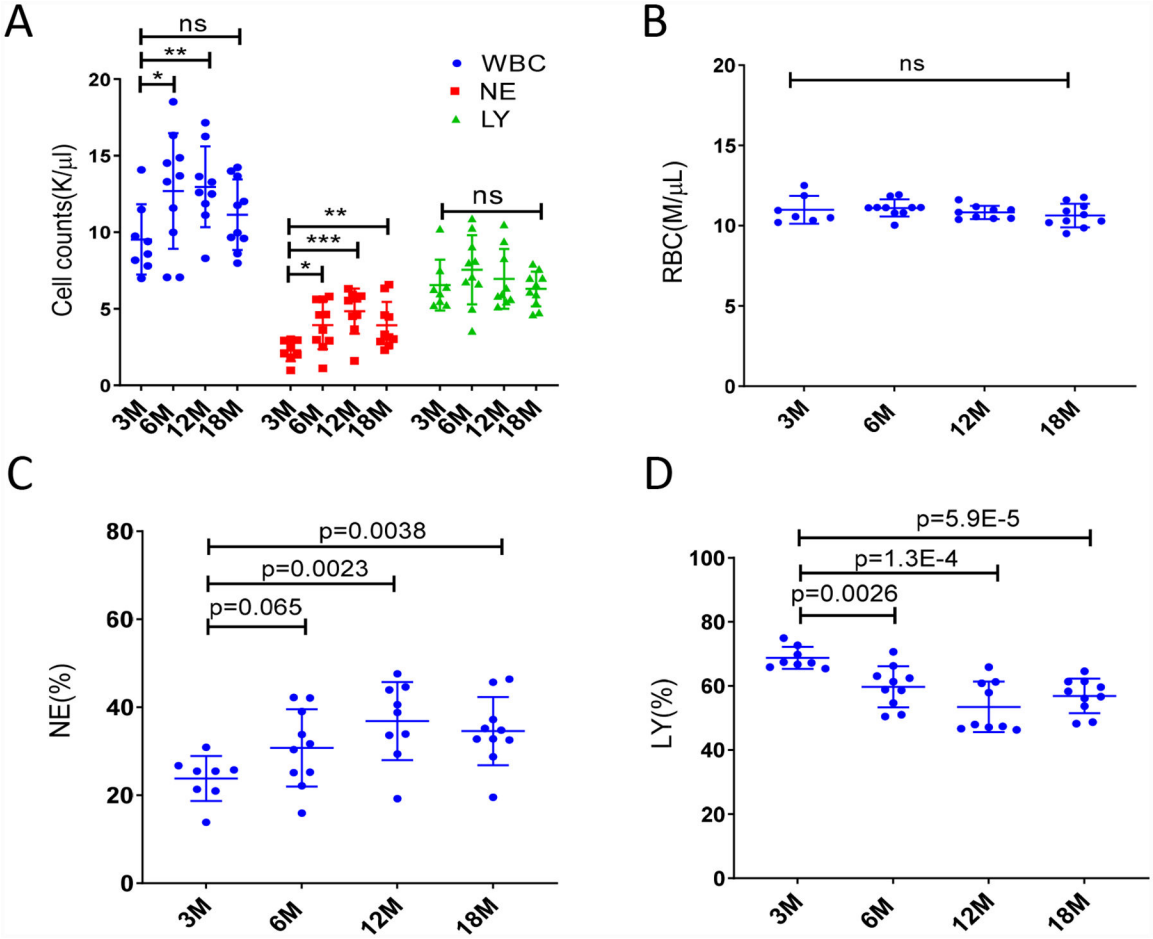

Figure 1.

The impact of aging on baseline hematological parameters in BALB/c mice. Peripheral blood from 3 to 18 month (M) old BALB/c mice collected from tail vein were subjected to five-part differential cell count analysis. **(A)** Total white blood cell (WBC), neutrophil (NE), lymphocyte (LY) counts. **(B)** Red blood cell counts. **(C, D)** The percentage of neutrophils (C) and lymphocytes (D). Significant values were determined by Student's T test (*P<0.05, **P<0.01). ns, not significant.

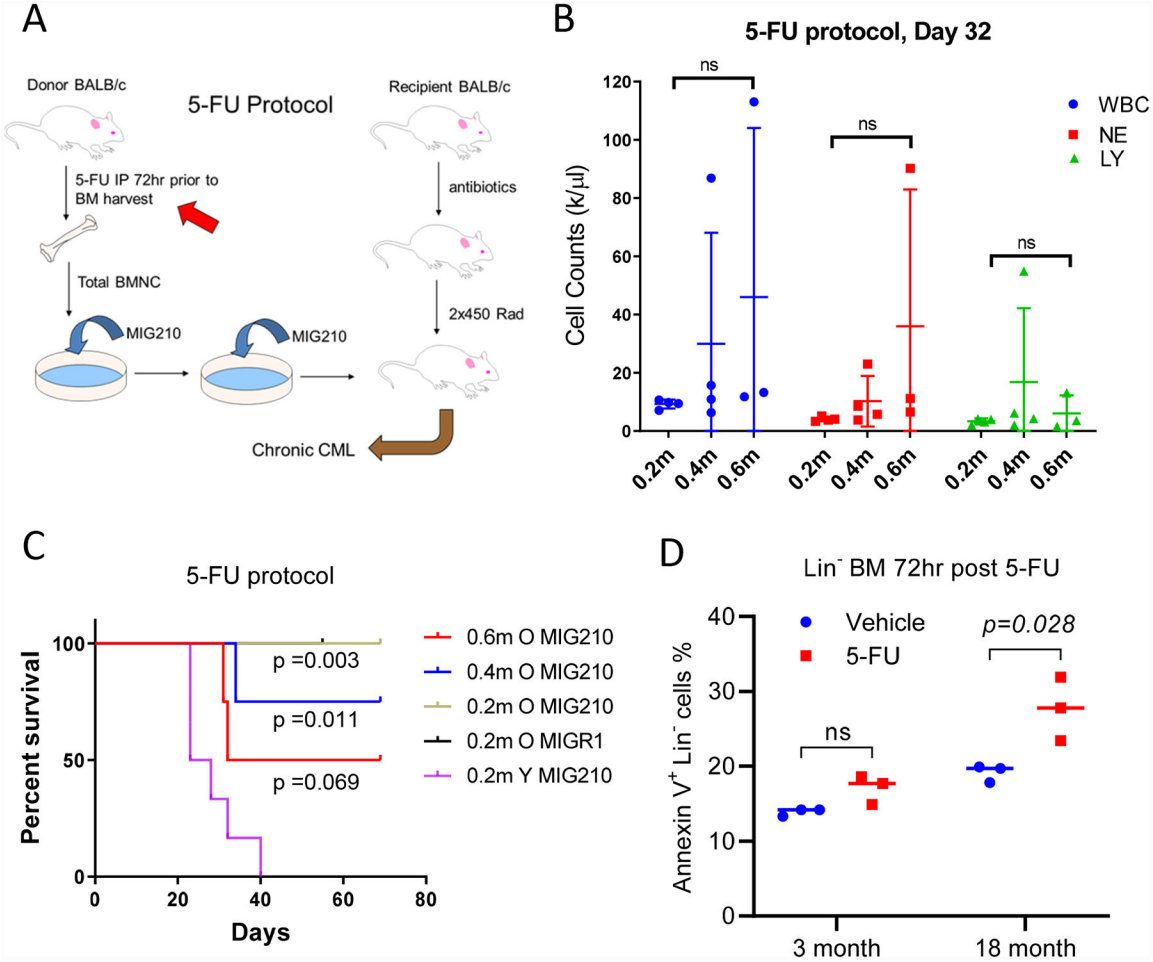

Figure 2.

The 5-FU myeloablation protocol was not suitable for CML induction in aging BALB/c mice. **(A)** Schematic presentation of the previously established 5-FU protocol for inducing CML in BALB/c mice. **(B)** Gradient dosage of either 0.1, 0.2, 0.4 or 0.6 million (m) of MIG210 transduced 18-month donor cells were transplanted into lethally irradiated BALB/c of the same age. Peripheral blood collected from the transplanted mice 32 days post BMT were analyzed for WBC, NE, and LY counts. One mouse in 0.6m group died of CML by the time of analysis. The 0.1m group failed to engraft and was not shown. **(C)** Kaplan-Meier survival curve of the transplanted mice for MIG210 or MIGR1 transduction and transplantation in 18-month (O) donor and recipient mice compared to that of MIG210 in 3-month (Y) mice. The p values were calculated against 0.2M Y MIG210 group. **(D)** 3-month and 18-month BALB/c mice were treated with vehicle or 200mg/kg 5-FU 72hr before BM harvest, and the percentage of apoptotic cells in lineage negative fractions of the BM nucleated cells were measured by flow cytometry with Annexin-V labeling. Three mice per group.

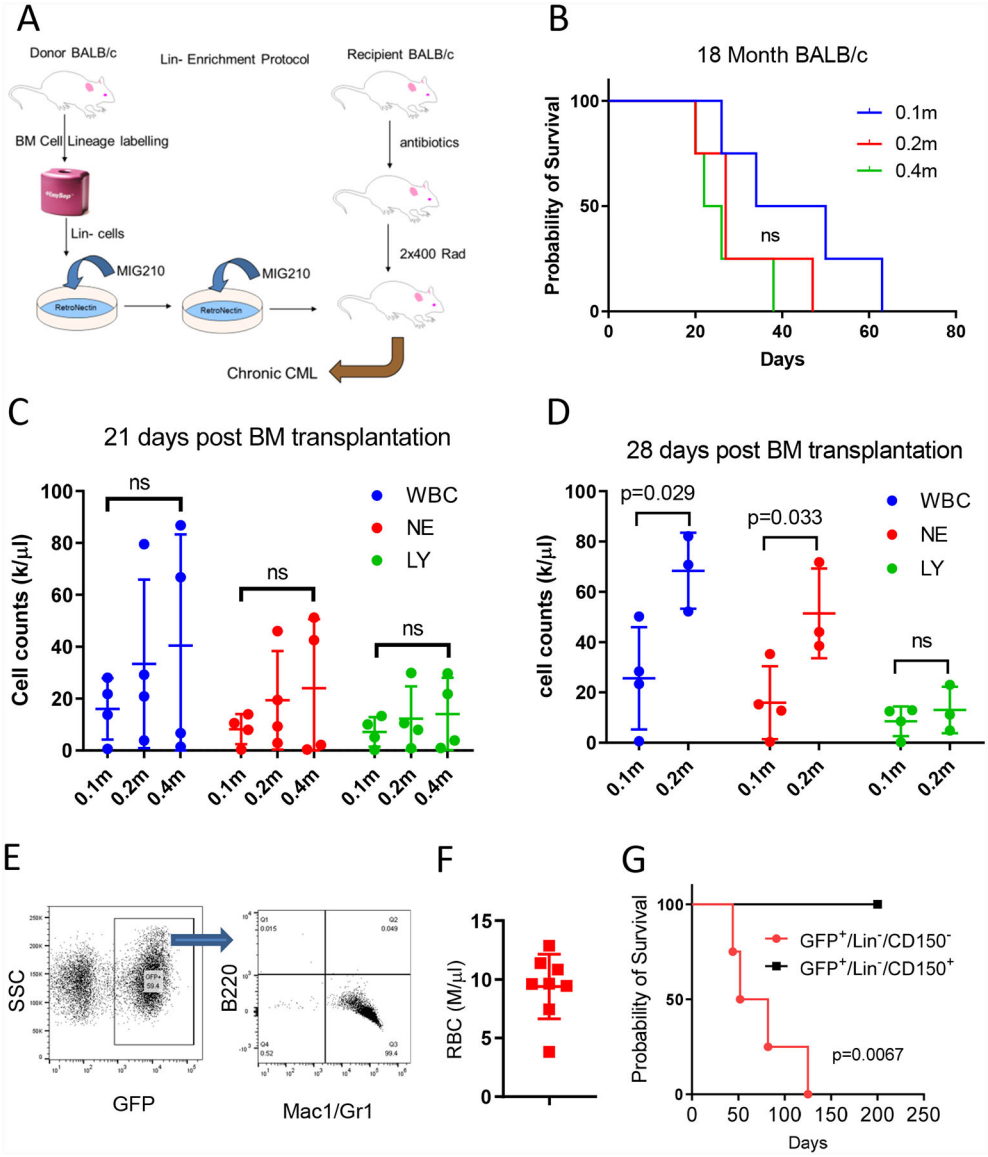

Figure 3.

Efficient CML induction in aging BALB/c mice by the immunomagnetic lineage depletion protocol. **(A)** Schematic presentation of the immunomagnetic lineage depletion protocol for generating CML in BALB/c. Gradient dosage of 0.1, 0.2, or 0.4 million of MIG210 transduced 18-month donor cells were transplanted into lethally irradiated BALB/c of the same age. **(B)** Kaplan-Meier plots for mouse survival post transplantation. **(C,D)** Peripheral blood collected from the CML mice at 3 (C) and 4 (D) weeks post transplantation were analyzed for WBC, NE, and LY counts. **(E)** Peripheral blood nucleated cells collected from the 0.2m diseased mice were analyzed for GFP and lineages. **(F)** RBC count of aging CML mice with 0.1m and 0.2m transduced cells. **(G)** Survival of secondary transplants of purified $GFP^{+}Lin^{-}$ CML cell fractions, 10,000 cells each, to 18-month old recipient mice. Statistical significance was determined by student's T test.

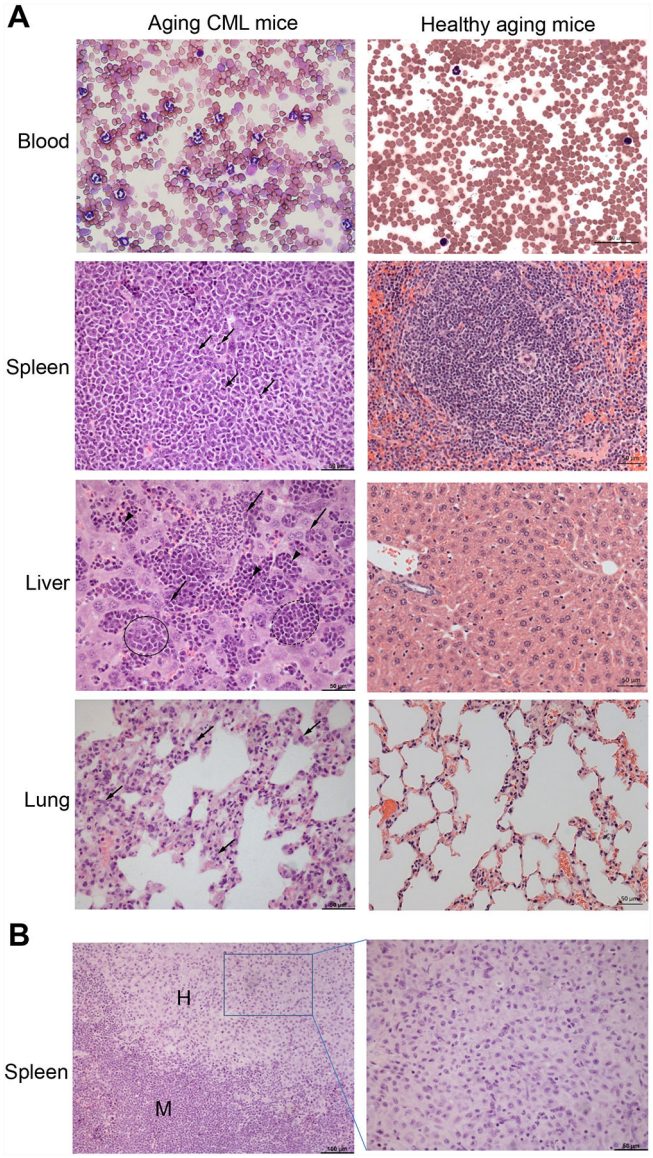

Figure 4.

Histopathology of CML in aging mice. **(A)** Tissue histopathology of aging CML mice (left panels): blood with excessive mature neutrophils, spleen with a sheet of myeloid leukemia cells containing numerous mitotic figures (arrows), hypercellular liver sinusoids due to the presence of immature myeloid (solid circle and arrows) and erythroid cells (dashed circle and arrowheads), and lung alveolar interstitium hypercellular with immature myeloid cells (arrows). Histology of a healthy 20-month aging mouse was shown for comparison (right panels). **(B)** Splenic histiocytic sarcoma (H) adjacent to an area infiltrated by myeloid leukemia cells (M).

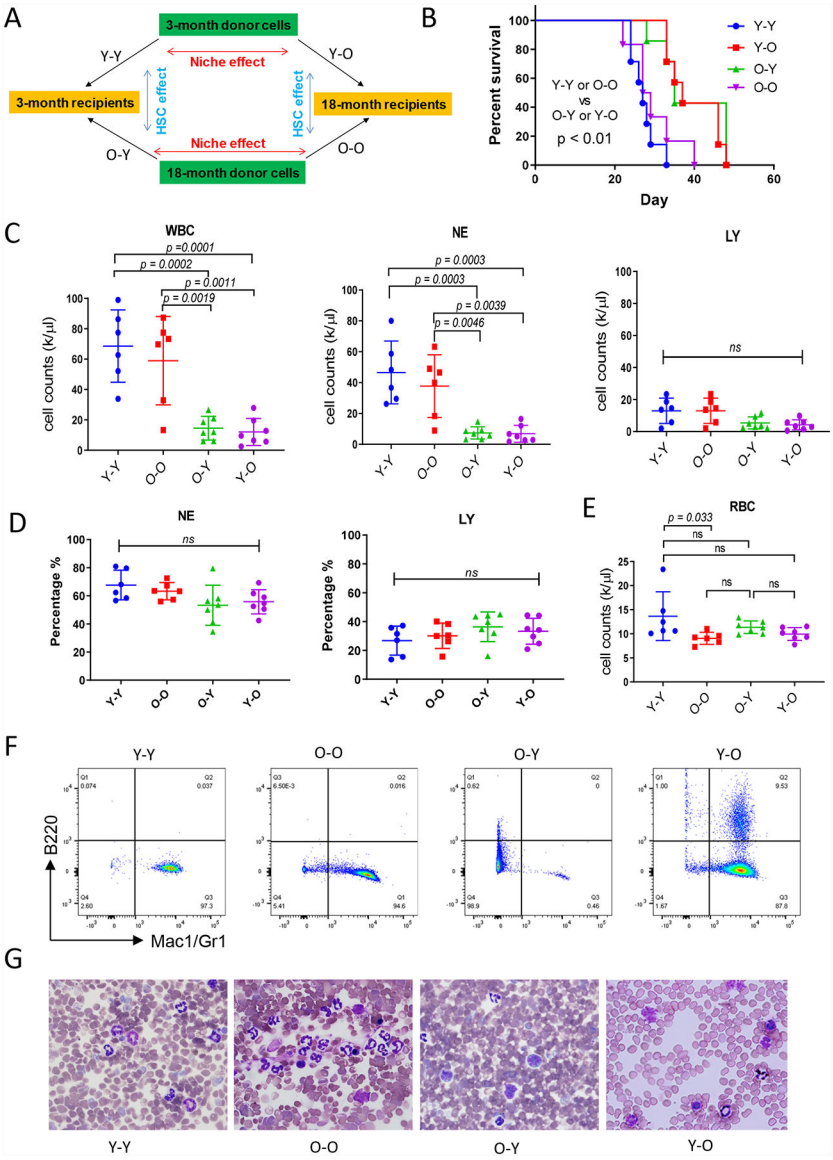

Figure 5.

Aging of both HSCs and bone marrow niche affected the pathogenesis of CML. **(A)** Schematic presentation of the cross-transplantation experiment in BALB/c mice. The aging HSC and niche effects were examined by transplanting either the MIG210 transduced young or old stem/progenitor cells into lethally irradiated young or old recipients. **(B)** The survival percentage post transplantation was plotted as Kaplan-Meier curves. **(C-E)** Peripheral blood cell counts collected from the CML mice at 4 weeks post transplantation were analyzed for WBC, NE, LY, and RBC counts and percentage. **(F,G)** Blood of the moribund mice was examined for lineage differentiation (F) and H&E staining of blood smear (G). Flow cytometry figures shown were gated from GFP^+ fractions of peripheral blood nucleated cells. A mouse with immature blood cells was shown for the O-Y group and a mouse with

mixed phenotype acute leukemia was shown for the Y-O group. Ordinary one-way ANOVA was used for statistical calculation in C-E.