Growth and Metastasis of Intraocular Tumors in Aged Mice

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PURPOSE. Since deterioration of the immune apparatus is closely associated with cancer, we examined the effect of aging on the growth and metastasis of intraocular melanomas in mice.

METHODS. Murine B16LS9 melanoma cells were transplanted into the posterior compartment of the eye (vitreous chamber) and intraocular tumor growth and development of liver metastases were evaluated in young (8–10 weeks of age) and old (>18 months of age) mice. Liver metastases were also induced by intrasplenic injection of melanoma cells. Natural killer (NK) cells from the livers of mice harboring liver metastases were evaluated in vitro for their cytolytic activity.

RESULTS. Tumors grew more rapidly in the eyes of young mice than old mice, yet old mice developed significantly more liver metastases. Increased liver metastasis in old mice was evident even when melanoma cells were injected intrasplenically as a means of bypassing the influence of the ocular immunosuppressive environment. Increased liver metastases in old mice correlated with reduced cytolytic activity of liver NK cells. Lethally irradiated young mice reconstituted with bone marrow from old donors developed significantly more liver metastases than young mice reconstituted with bone marrow from young donors, indicating that bone marrow–derived cells were the root cause of the heightened development of metastases in old mice.

CONCLUSIONS. Aging affects the growth and metastasis of intraocular melanomas. Even though intraocular melanomas grow slower in old mice, the development of liver metastases is exacerbated and correlates with a reduction in liver NK cell activity in the old mouse.

Keywords: aging, eye, ocular tumors, myeloid-derived suppressor cells, metastases, NK cells, uveal melanoma

Aging is associated with a widespread deterioration of the immune apparatus and an increased risk for cancer. Both the adaptive and innate immune responses degrade with age.[1–3] A number of conditions contribute to the diminished immune function associated with aging. These include thymic involution, which leads to decreased numbers of T and B lymphocytes,[4] reduced T-cell lymphoproliferative responses,[5] defects in antigen-presenting cell function,[6] and impaired natural killer (NK) and NKT cell function.[2]

Uveal melanoma (UM) is the most common primary intraocular tumor in adults with an incidence in the United States of approximately four cases per million people per year.[7] About half of the patients with UM will develop metastases with the liver being the most commonly affected organ in up to 95% of the cases.[8] The mortality rate in the 15 years following diagnosis hovers at approximately 50% and has not improved in the past 50 years.[9,10] Moreover, there is no currently available therapeutic modality that has been shown to prolong the lives of UM patients with liver metastases.[11] One of the most exciting areas of cancer research is the emergence of immune-based therapies that have produced promising results.[12–17] Developing new treatments for UM patients, such as immunotherapy, would benefit enormously from prospective studies in animal models. Historically, three categories of animal models have been used in ocular melanoma research: transplantation of human UM cells into the eyes of immune-deficient mice such as

T-cell–deficient nude mice[18,19]; in situ transformation of ocular cells through the insertion of an oncogenic transgene into inbred mouse strains[20,21]; or transplantation of cutaneous murine melanoma cells, such as B16 melanoma, into the eyes of syngeneic C57BL/6 mice. Transplantation of human UM cells into the eyes of immune-deficient mice produces intraocular melanomas that metastasize to the liver and, thus, recapitulates the metastatic behavior of UM.[22–27] However, because this is a tumor xenograft residing within an immune-deficient host, this model has limited applications and it is not amenable for studying the role of the adaptive T-cell–mediated antitumor response. Murine models of in situ transformation of ocular cells through insertion of an oncogenic transgene into the genome of inbred mice have been fraught with confounding properties that limit their usefulness. Our laboratory produced a transgenic mouse model using the simian virus 40 (SV40) transgene under the influence of a tyrosinase promoter.[20] Pigmented intraocular tumors arose at the choroid-RPE interface. However, the tumors displayed ultrastructural and morphological characteristics consistent with RPE carcinomas. Moreover, pigmented cutaneous tumors arose in a high percentage of the transgenic mice, further complicating its utility as a model of UM. More recently, Schiffner et al.[21] reported a transgenic mouse model in which the glutamate receptor 1 (Grm1) transgene, under the control of the dopachrome tautomerase promoter, was introduced into

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C57BL/6 mice. Transgenic mice developed melanocytic tumors localized in the ciliary body and choroid, but also in the skin. In an earlier study in this transgenic model, skin melanoma cells were detected in lymph nodes, spleen, lung, and liver.[28] Because the skin and ocular tumors both express the Grm1 transgene, it is not possible to distinguish skin metastases from UM metastases in this model. A third category of animal models of UM involves the intraocular transplantation of cutaneous B16 murine melanoma into the eyes of syngeneic C57BL/6 mice. The most obvious shortcoming of this approach is that skin melanomas differ markedly from UM in their molecular and clinical properties. However, the B16 melanoma arose in a C57BL/6 mouse and thus confronts the host only with tumor-associated and tumor-specific antigens and as a result allows analysis of the adaptive and innate immune responses. Moreover, B16LS9 melanomas display a propensity to metastasize to the liver, which recapitulates the metastatic behavior of human UM. Prospective studies using this model have revealed a strong association between NK cell activity and resistance to the development of liver metastases that is remarkably similar to findings from retrospective studies in human UM patients.[19,23,25,29–35]

It bears noting that the liver has the highest concentration of NK cells of any organ in the body[36] and a large body of evidence strongly implicates NK cells in the resistance to UM metastases.[37] Studies using human and murine NK cells have shown that uveal melanoma cells are susceptible to NK cell–mediated cytolysis in vitro.[19,34,38] In vivo evidence reinforces the notion that NK cells act to control the development of liver metastases arising from intraocular melanomas in mice.[22,23,25,27,32] The susceptibility of UM cells to NK cell–mediated cytolysis is inversely correlated with the expression of MHC class I molecule expression on the tumor cells.[19,34] The resistance of melanoma cells to cytolysis by NK cells is based on the capacity of major histocompatibility complex (MHC) class I molecules to transmit an "off" signal to NK cells and arrest their cytolytic activities. This phenomenon is the basis for the "missing-self" hypothesis, which posits that NK cells are programmed to kill any mammalian cell failing to express MHC class I molecules.[39] Support favoring a role for the missing-self hypothesis in the development of liver metastases in UM patients was reported in a study by Verbik et al.,[35] who found that UM liver metastases displayed a 10-fold higher expression MHC class I molecules compared with the MHC class I expression on the primary UM cells from the same patient.

The present study examined the effect of aging on the growth of intraocular tumors, development of liver metastases, and NK cell activity in the liver. The rationale for these investigations is based on the following: UM is the most common primary intraocular malignancy in adults, the liver is the most common site for metastases arising from intraocular melanomas, NK cells are arguably the most important immune element that limits the development UM liver metastases, and innate immune responses such as NK cell activity deteriorate with age. We used the current mouse model, being fully aware of both its shortcomings and advantages and we tempered our interpretation of the results accordingly.

MATERIALS AND METHODS

Cell Lines

The B16LS9 cutaneous murine melanoma cell line was kindly provided by Hans E. Grossniklaus (Emory University School of Medicine, Atlanta, GA, USA). Murine B16LS9 cells were derived from hepatic metastases originating from posterior compart-

ment inoculation of B16-F1 cutaneous melanoma cells in C57BL/6 mice.[40] Tumor cells were maintained in complete Dulbecco's modified Eagles' medium containing 10% fetal bovine serum (HyClone, Logan, UT, USA), 100 U/mL penicillin, 50 ng streptomycin, 0.1% Fungizone (BioWhittaker, Walkersville, MD, USA), 2.0 mM glutamine (BioWhittaker), 0.01 M HEPES buffer (BioWhittaker), and 0.5% 2-Mercaptoethanol (Sigma-Aldrich Corp., St. Louis, MO, USA).

Mice

Young C57BL/6 mice (8–10 weeks of age) were obtained from the animal colony at the University of Texas Southwestern Medical Center (Dallas, TX, USA). Old C57BL/6 mice (>18 months of age) were purchased from the National Institute on Aging (Bethesda, MD, USA). All mice were maintained in a dedicated pathogen-free environment. All animals were housed and cared for in accordance with the guidelines of the Institutional Animal Care and Use Committee of the University of Texas Southwestern Medical Center and the ARVO statement concerning the Use of Animals in Ophthalmic and Vision Research. Tumor-containing eyes were enucleated when the tumor-containing eyes reached 4.0 mm in diameter. No intraocular tumors reached a size that would perforate the eye. All surgical procedures and intrasplenic injections were performed under anesthesia in the form of a cocktail of ketamine and xylazine given intraperitoneally and buprenorphine was given subcutaneously as an analgesic after splenic injections and enucleations.

Anterior Chamber Tumor Injections

Murine B16LS9 melanoma cells (1×10^5) were injected into the anterior chamber (AC) of the eye as described elsewhere.[41] Tumor growth in the AC was observed under a dissecting microscope and tumor volume was scored as a percentage of the AC occupied by tumor.[42]

Posterior Compartment Tumor Injections

Murine B16LS9 melanoma cells were injected intravitreally into the posterior compartment (PC) of the eye as described previously.[22] Intraocular melanomas that form after these injections disseminate from the eye and form liver metastasis.[23,43] Murine B16LS9 tumor cells (5×10^4) were injected into the PC and tumor-bearing eyes were enucleated when they reached 4.0 mm in diameter. Mice were euthanized 2 weeks after enucleation and their livers were collected for histologic analysis. Tumor volumes were assessed on day 14, as this is the time point at which the tumors in the eyes of most young mice necessitated enucleation to avoid rupture of the globe. Hematoxylin and eosin (H&E)-stained sections of tumor-bearing eyes were assessed by three masked observers and the tumor volume was calculated using the formula: volume = length $\times$ width squared.[44] Tumor volume was also assessed using Zeiss AxioVision SE 64 Rel 4.9.1 software (Jena, Germany).

Intrasplenic Tumor Injections

Intrasplenic tumor cell injection is an alternative method to produce liver metastases by facilitating the dissemination of tumor cells to the liver via the hepatic portal circulation.[45,46] Melanoma cells (5×10^4) were injected beneath the spleen capsule of anesthetized mice. Mice were euthanized 14 days later and their livers collected for further analyses.

Assessment of Liver Metastases

Liver metastases arising from intraocular injections of B16LS9 melanoma tumor cells were assessed by histology. The left lobes of formalin-fixed livers were sectioned at 100-μm intervals, stained with an H&E staining method, and examined in a masked fashion by three independent observers. The results were reported as the mean number of metastases per 10 random low-power fields (×100). Liver metastases arising from intrasplenic injection of B16LS9 melanoma tumor cells were assessed by counting surface tumor nodules on the liver by both visual gross observation and counting the number of metastatic foci using a dissecting microscope.[47]

In Vitro NK Cell–Mediated Cytotoxicity Assay

Liver cells were isolated and enriched for NK cells with a CD49b Positive Selection Kit (Stemcell Technologies, Vancouver, BC, Canada). The NK cell–enriched suspensions were examined by flow cytometry for purity using anti-NK1.1 antibody (BD Biosciences, San Jose, CA, USA) and anti-CD8 monoclonal antibody (BD Biosciences) and were found to be 74% NK1.1+ and less than 4% CD8+. A tritiated thymidine-based cytotoxicity assay was used to quantify NK cell–mediated cytolysis of tumor cells.[48] Purified liver NK effector cells (E) were co-cultured with B16LS9 target cells (T) labeled with ^{3}H-thymidine (Perkin Elmer, Waltham, MA, USA) at an E:T ratio of 25:1 for 18 hours at 37°C. Cells were harvested with a Combi Cell Harvester (SKATRON, Lier, Norway) according to manufacturer's instructions. Radioactivity was measured in a liquid scintillation counter. Cytotoxicity was calculated using the following formula: Percent Cytotoxicity = ([A−B]/A) × 100, where *A* represents counts per minute (CPM) of tumor cells cultured alone, and *B* represents the CPM in test cultures.

Liposome-Encapsulated Dichloromethylene Diphosphonate (Clodronate)

Multilamellar liposomes were prepared as described earlier.[49] Briefly, 8 mg cholesterol and 86 mg phosphatidylcholine (Sigma-Aldrich Corp.) were dissolved in 10 mL chloroform (Sigma-Aldrich Corp.) in a round-bottomed flask. After low-vacuum rotary evaporation at 37°C, a thin film was formed on the inner surface of the flask. This film was then dispersed by gentle rotation overnight in PBS for the preparation of PBS-containing liposomes (PBS-LIP). For clodronate liposomes (C12MDP-LIP), 2.5 g clodronate (C12MDP; Roche, Mannheim, Germany) was dissolved in 10 mL PBS. The suspension was sonicated for 3 minutes at 20°C. To remove free C12MDP, the liposomes were washed twice by centrifugation in PBS at 10,000*g* for 30 minutes and resuspended in 4 to 10 mL of PBS that contained approximately 20 mg C12MDP. Each 100 μL C12MDP-LIP suspension contained 0.2 mg C12MDP. The cytotoxicity of C12MDP-LIP and PBS-LIP was tested using an in vitro toxicity assay using RAW 264.7 macrophages as target cells. Within 24 hours, C12MDP-LIP (100 μL) typically induced 85% to 90% cytotoxicity of 1×10^5 RAW 264.7 cells. Liposomes were used immediately, and excess liposomes were stored at 4°C for up to 1 month, after which, the liposomes were discarded.

Macrophage Depletion

Previous studies have shown that subconjunctival injection of C12MDP-LIP (clodronate-containing liposomes; Sigma-Aldrich Corp.) induces the elimination of more than 95% of the conjunctival macrophages[50] and more than 99% depletion of F4/80+ macrophages that infiltrate intraocular Ad5E1 tumors.[51]

Under an operating microscope, the conjunctiva was lifted and the C12MDP-LIP suspension (6 μL) was injected into the bulbar conjunctiva using a 30-gauge needle mounted on a 1-mL tuberculin syringe. Injection of the C12MDP-LIP suspension resulted in a bleb around the injection site. To obtain a more equal distribution of the suspension around the limbus, the dose was divided by injecting at three different sites 120° apart around the limbus until a circular conjunctival bleb was obtained. Phosphate-buffered saline–LIP was used as a negative control for macrophage depletion. Liposome injections were performed on the day of tumor injection and repeated every 3 to 4 days throughout tumor observation.[41] Although we did not confirm macrophage depletion in the tumor-bearing eyes in the present study, we should emphasize that this protocol for deleting macrophages has been used previously by two independent laboratories using two different intraocular tumor models in mice and has been shown to effectively delete macrophages within intraocular tumors.[41,48,51,52]

Generation of Bone Marrow Chimera Mice

Young C57BL/6 wild-type (WT) mice (8–10 weeks old) were lethally γ-irradiated (900 cGy) and reconstituted with bone marrow cells from the femur and tibia of either old (>18 months old) or young (8–10 weeks old) C57BL/6 WT mice. Bone marrow cells (2×10^7) from donor mice were injected intravenously into each recipient mouse. Chimeric animals were allowed to recover for 8 weeks before intrasplenic injection of B16LS9 tumor cells as described above.

Flow Cytometry

Liver-infiltrating leukocytes were isolated by isotonic Percoll centrifugation (900*g*, 25 minutes). Red blood cells were lysed using ACT lysis buffer (NH_4Cl, 140 mM; $C_4H_{11}NO_3$, 17 mM; pH 7.2). The leukocytes were resuspended in fluorescence-activated cell sorter (FACS) medium (1 × PBS, 2 mM EDTA, 0.5% BSA, 0.09% sodium azide). Then, 1×10^6 cells of each sample were stained using the following antibodies: FITC-conjugated anti-NK1.1, allophycocyanin (APC)-conjugated anti-Gr-1 and phycoerythrin-conjugated anti-CD11b were from BD Pharmingen (San Diego, CA, USA). Phycoerythrin-Cy5-conjugated anti-CD3 was purchased from Tonbo Biosciences (San Diego, CA, USA). After staining procedures, cells were washed twice with FACS medium, and analyzed on the Attune NxT acoustic focusing cytometer (Applied Biosystems; Life Technologies, Waltham, MA, USA). The results were analyzed using FlowJo v10 software (FlowJo, LLC, Ashland, OR, USA).

RESULTS

Delayed Growth of Intraocular Tumors in Old Mice

Although both innate and adaptive immune responses deteriorate with advancing age,[1,2,6] intraocular tumors reside in an immune-privileged site in which immune surveillance is either excluded or diminished. Accordingly, we examined the growth of melanoma cells transplanted into the vitreous cavity of old and young mice. This route of injection was initially selected because melanoma cells injected into the vitreous cavity of the mouse eye invade the uveal tract and preferentially metastasize to the liver.[23,31,43] The results of multiple experiments indicated that tumors grew more swiftly in the eyes of young mice than old mice (Figs. 1A–C).

To determine if either adaptive or innate immune responses influenced the growth of intraocular melanoma, B16LS9 tumor cells were injected into the AC of NOD-SCID mice that lack T cells and thus cannot mount adaptive immune responses and
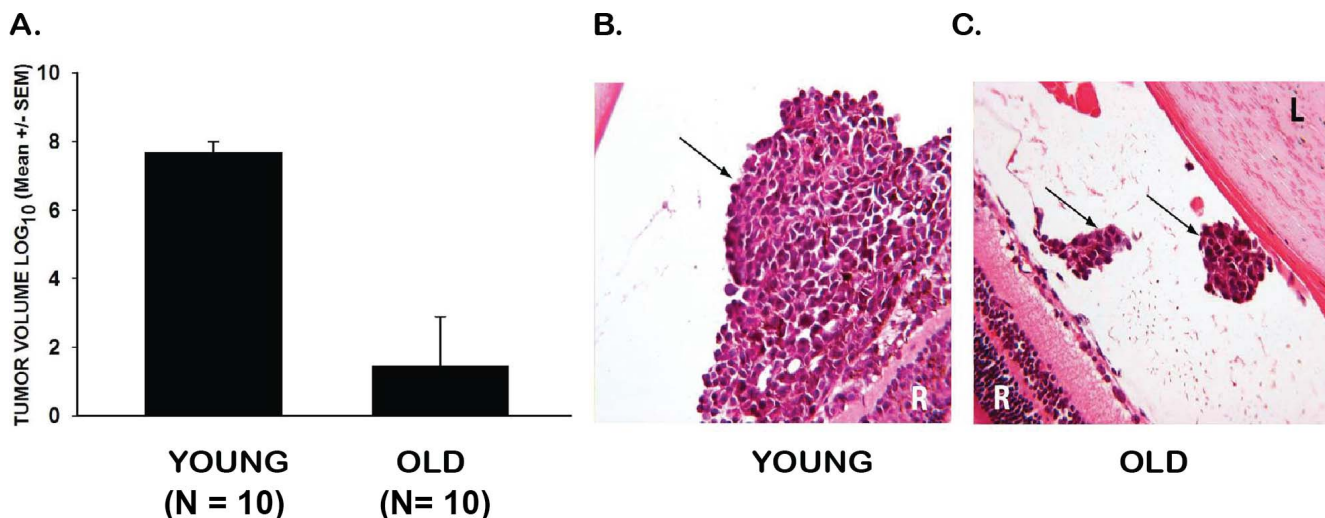
FIGURE 1. Growth of intraocular melanomas in old and young mice. (**A**) Tumor volume in eyes of old (>18 months) and young (8–10 wks) mice 14 days after intravitreal injection of B16LS9 melanoma cells. (**B**) Intraocular tumor (*arrow*) in young mouse at day 14 and (**C**) in old mouse at day 14. R, retina; L, lens. This experiment was performed three times with similar results. $n = 10$ mice/group/experiment.

tumor growth was compared with WT mice with an intact T-cell repertoire. For these experiments, we chose to inject tumors into the AC, as these tumors were readily visible in the AC and allowed us to make regular observations and measurements of intraocular tumor volume on a day-to-day basis. By contrast, we are not able to gauge tumor volume in the PC tumors until the eyes are enucleated for histology. Using the AC tumor model, we found that the absence of adaptive immune response did not lead to accelerated intraocular tumor growth in T-cell–deficient NOD-SCID mice (Fig. 2). The effect of the innate immune system on intraocular tumor growth was examined by treating WT mice with anti-asialo GM1 antiserum as a means of disabling NK cell activity. Although anti-asialo GM1 antiserum causes steep inhibition of NK activity, it had no effect on the tempo of intraocular tumor growth compared with untreated WT mice (Fig. 2).

It has been reported that depletion of ocular macrophages in aged mice results in a profound reduction in intraocular melanoma growth in the ACs of old, but not young mice.[52] With this in mind, we depleted ocular macrophages using clodronate-containing liposomes.[48,52,53] However, instead of injecting melanoma cells into the AC, B16LS9 melanoma cells were injected into the vitreous cavity of young and old mice as before, and the eyes were enucleated 14 days later and tumor volume assessed by compound microscopic (Carl Zeiss Microscope, Jena, Germany) examination of histopathological specimens using image analysis software (Zeiss AxioVision SE 64 Rel 4.9.1; Jena, Germany). The results of three independent experiments revealed that depletion of ocular macrophages did not affect the growth of intraocular tumors in either old or young mice (Fig. 3). The results also showed that, as before, tumors grew slower in the vitreous cavity in old mice than they did in young mice.
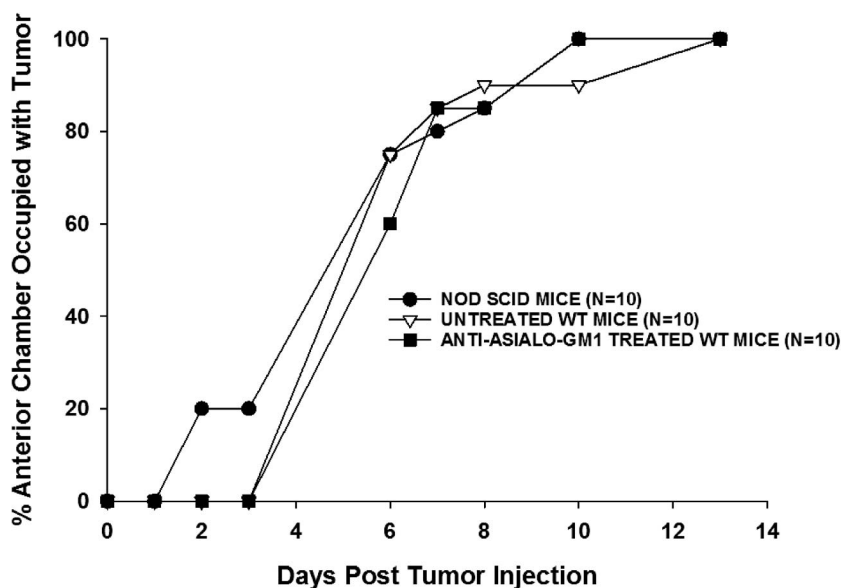

FIGURE 2. Growth of melanoma cells injected into the AC of T-cell–deficient mice and NK-cell–deficient mice. Growth of B16L69 melanomas in the AC of T-cell–deficient NOD-SCID mice, WT mice depleted of NK cells using anti-asialo GM1 antiserum, and untreated WT mice. There were 10 mice per group. This experiment was performed once.
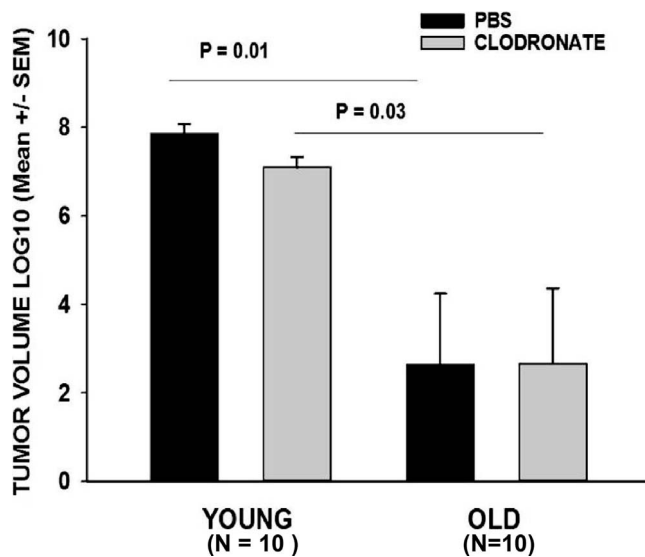
FIGURE 3. Effect of macrophage depletion on the growth of intraocular melanomas in old and young mice. Murine B16LS9 melanoma cells were injected intravitreally on day 0. Ocular macrophages were depleted by subconjunctival injections of clodronate-containing on the day of tumor injection and every 3 to 4 days throughout the tumor observation period. Control mice were similarly treated using PBS-containing liposomes. There were 10 mice/group/experiment.

Liver Metastases in Old and Young Mice

The swifter growth of intraocular tumors in young mice suggested that these mice would develop more extensive liver metastases than old mice. Accordingly, we injected B16LS9 melanoma cells into the vitreous cavity of young and old mice and enucleated the tumor-bearing eyes 10 days later. Mice were euthanized 14 days after enucleation and liver metastases were counted. The results revealed that even though intraocular tumors grew slower in old mice, there was a more than 3-fold increase in the numbers of liver metastases in old mice compared with their young counterparts (Fig. 4A). Additional experiments used an alternative model for inducing liver metastases to confirm that the increased number of metastases in old mice was due to the age of the host and not the result of the intraocular milieu from which the tumors disseminated. Accordingly, liver metastases were induced by intrasplenic injection of tumor cells. This technique bypasses the influence of the intraocular environment and promotes the development of liver metastases by directing the tumor cells into the hepatic portal circulation. The results of multiple experiments revealed that liver metastases were significantly higher in old mice than young mice (Figs. 4B–D).

Depressed Liver NK Cell Activity in Old Mice

A compelling body of evidence suggests that NK activity exerts a profound effect in controlling the development of liver metastases of intraocular melanomas in humans and mice.[37,54,55] We and others have previously reported that NK cells in the liver significantly inhibit the development of liver
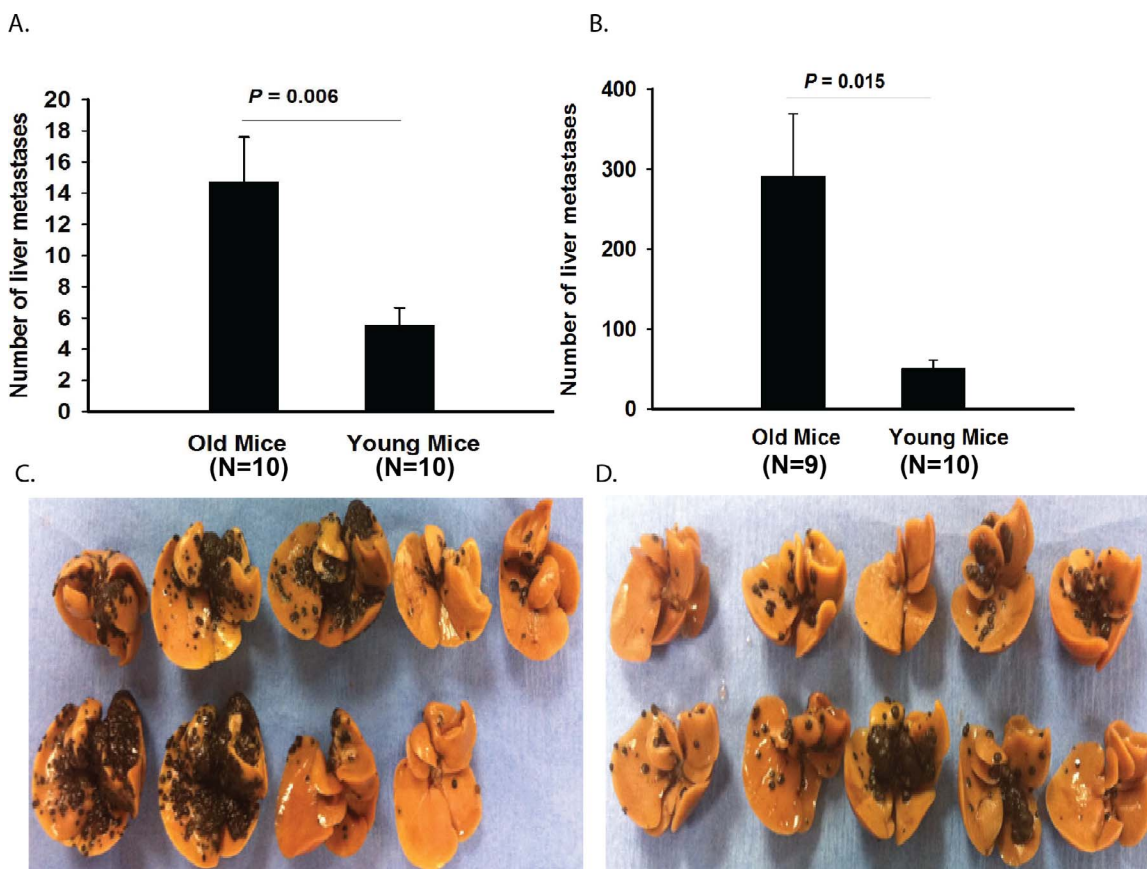

FIGURE 4. Liver metastases in old and young mice. (**A**) Number of metastases arising following intravitreal injection of B16LS9 melanoma cells or (**B**) following intrasplenic injection of B16LS9 melanoma cells. (**C**) Photograph of livers from old mice and (**D**) young mice injected intrasplenically with B16LS9 melanoma cells. This experiment was performed twice with similar results. There were 10 mice/group/experiment.
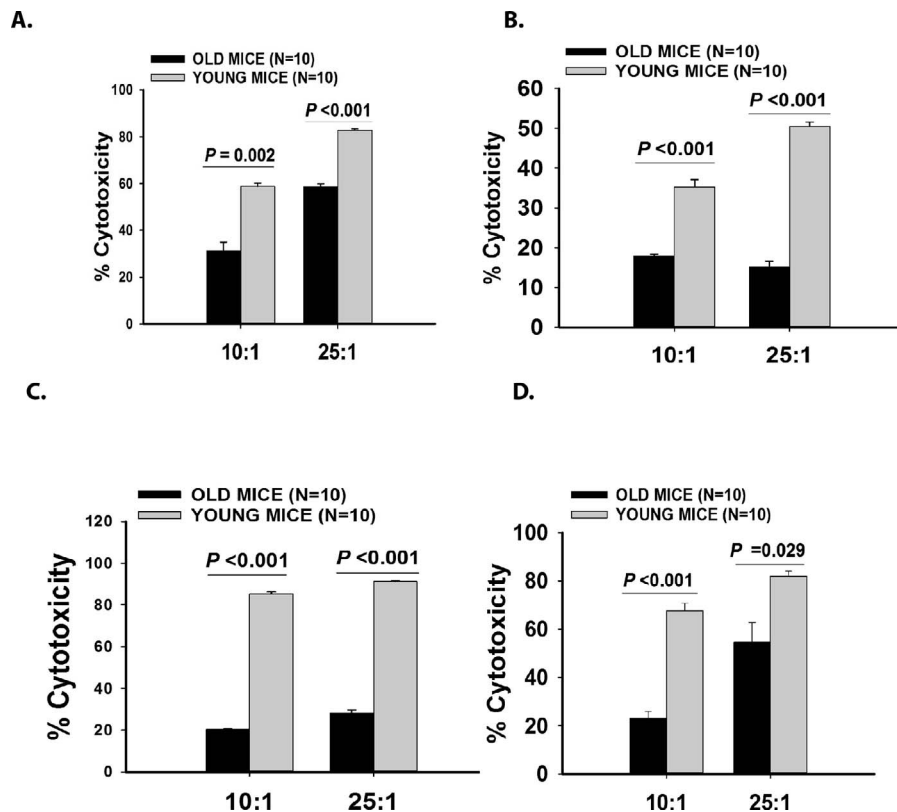
FIGURE 5. Liver NK cytotoxicity in naïve and tumor-bearing mice. (**A**) Natural killer cytotoxicity using liver mononuclear cells from naïve mice and Yac-1 target cells and (**B**) B16LS9 target cells. (**C**) Natural killer cytotoxicity using NK1.1$^+$CD3$^-$ NK cells enriched from liver mononuclear cells isolated from mice with liver metastases induced by intrasplenic injection of B16LS9 melanoma cells and tested against Yac-1 target cells and (**D**) tested against B16LS9 target cells. There were 10 mice/group/experiment.

metastases arising from intraocular melanomas in mice.[22,23,25,27,32,43] Accordingly, we evaluated the NK cell activity in the livers of mice with metastases. In vitro cytolysis of NK-sensitive YAC-1 lymphoma target cells and B16LS9 melanoma cells was significantly higher in young mice compared with old mice (Figs. 5A, 5B). Similar results were observed using purified (NK1.1$^+$CD3$^-$) NK cells (Figs. 5C, 5D).

The in vitro assays for measuring NK cytolytic activity used the same numbers of liver NK cells isolated from young and old mice. Thus, the enhanced NK cytolytic activity found in the livers of young mice was due to increased NK cell–mediated cytolysis on a per-cell basis, as the same number of NK effector cells from young and old mice were used in the in vitro assays.

We next entertained the hypothesis that young mice had increased numbers of liver NK cells compared with old mice. Lymphocytes isolated from the livers of non–tumor-bearing old and young mice were analyzed by flow cytometry for the number of NK1.1$^+$CD3$^-$ NK cells. Normal, non–tumor-bearing young mice had more than twice as many NK1.1$^+$ CD3$^-$ NK cells as old mice (Figs. 6A, 6B, 6E). There were also significantly higher total numbers of NK cells in young mice harboring liver metastases compared with old mice (Figs. 6C, 6D, 6F). Thus, young mice have both larger numbers of liver NK cells and greater liver NK cytolytic activity on a per-cell basis compared with old mice.

Bone Marrow From Old Mice Converts Young Mice to an Old Mouse Phenotype

The diminished cytotoxicity of liver NK cells in old mice could be due to either the direct effect of aging on NK cell precursors in the bone marrow or to the indirect result of the milieu in the liver created by non–bone-marrow–derived parenchymal cells. In the mouse, NK cells are derived from bone marrow precursors and have a half-life of 17 days or less depending on the technique used to assess NK cell turnover.[56,57] To determine if aging affects the bone marrow–derived NK cell precursors, bone marrow chimeric mice were generated by lethally irradiating young mice and reconstituting them with bone marrow from either young or old mouse donors. Eight weeks after bone marrow reconstitution, mice were challenged with intrasplenic injections of melanoma cells and the development of liver metastases was evaluated 14 days later. Young mice reconstituted with bone marrow from old donors developed a 3-fold higher number of liver metastases than the normal young control mice (Fig. 7A). By contrast, young mice reconstituted with bone marrow from young donors developed the same number of liver metastases as young control mice, indicating that the lethal irradiation and bone marrow reconstitution itself did not account for the increased number of metastases in young mice reconstituted with bone marrow from old donors (Figs. 7B–D).

Differential Expression of Myeloid-Derived Suppressor Cell Populations in Old and Young Mice

Myeloid-derived suppressor cells (MDSCs) are broadly defined as CD11b$^+$Gr1$^+$ cells.[58] Myeloid-derived suppressor cells are increased in cancer patients and tumor-bearing animals and are associated with depressed innate and adaptive immune responses.[59,60] Subsets of MDSCs include granulocytic (Gr1^{high}) and monocytic (Gr1^{low}) populations. In many
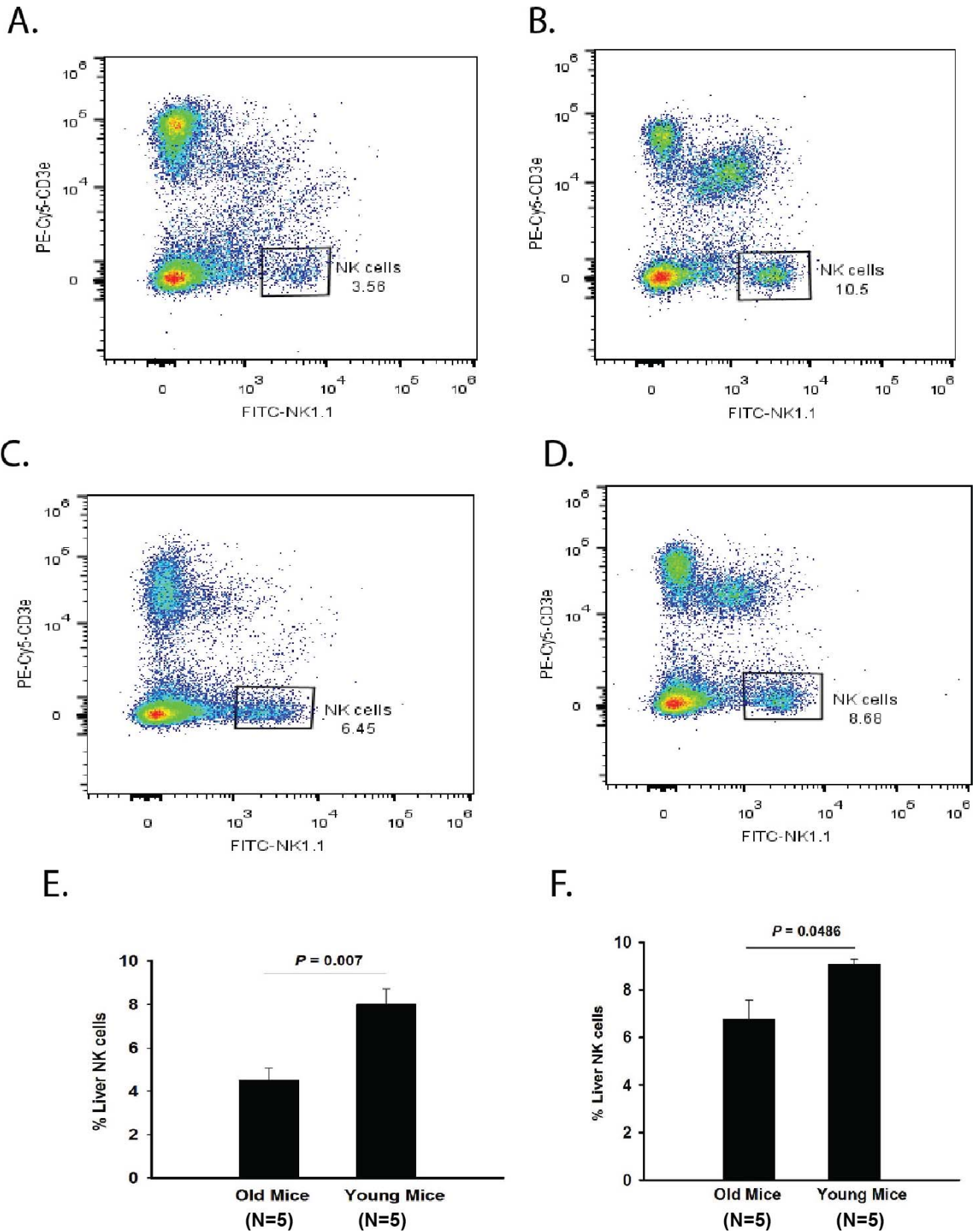
FIGURE 6.   Number of NK cells in the livers of (A) old naïve mice, (B) young naïve mice, (C) old mice with liver metastases induced by intrasplenic injection of B16LS9 melanoma cells, and (D) young mice injected intrasplenically with B16LS9 melanoma cells. (E) Number of NK cells from naïve mice and old mice and (F) young and old mice with liver metastases. This experiment was performed three times with similar results. There were five mice/group/experiment.
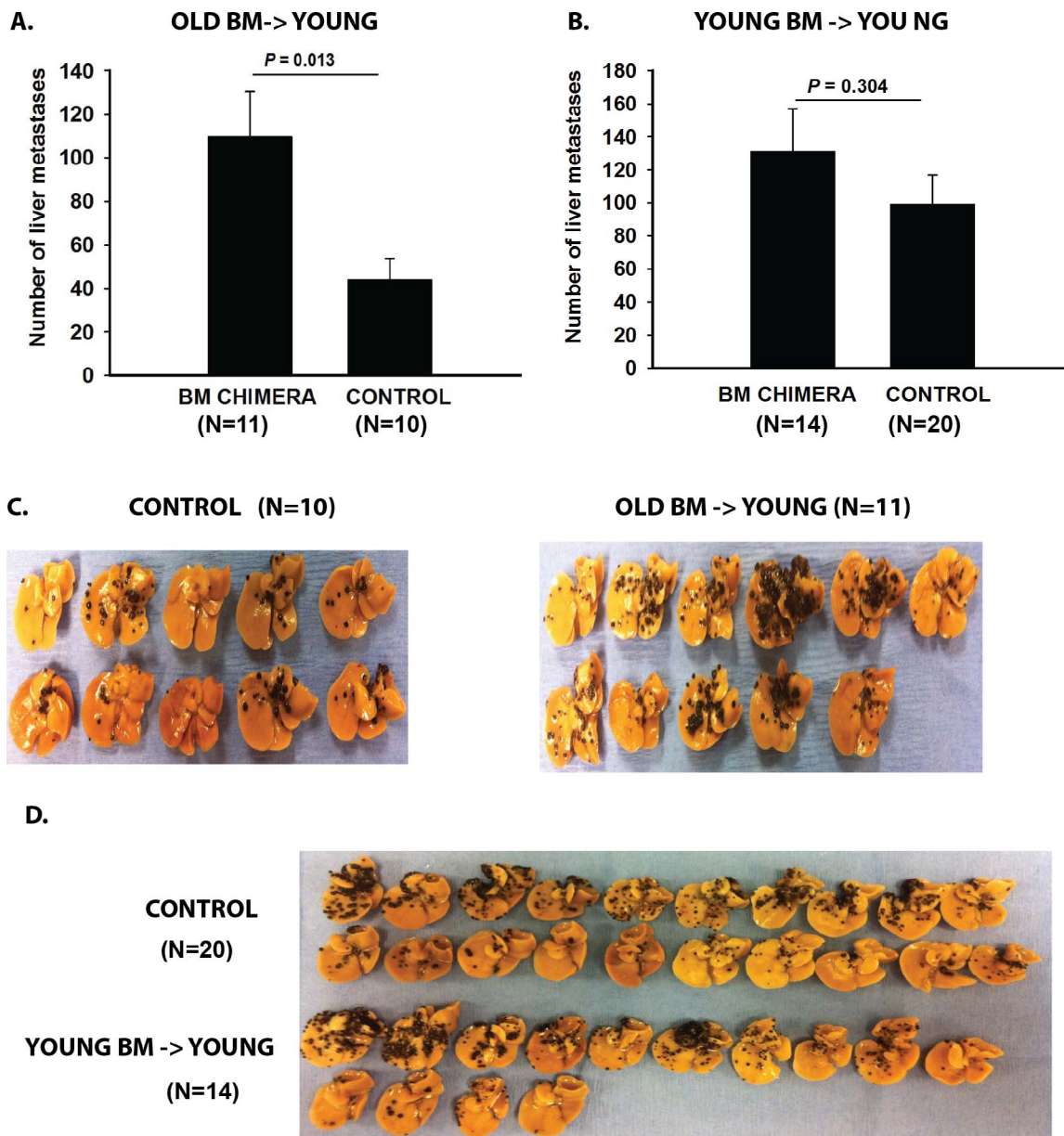
A. **OLD BM-> YOUNG**

B. **YOUNG BM -> YOUNG**

C. **CONTROL (N=10)**

OLD BM -> YOUNG (N=11)

D.

CONTROL (N=20)

YOUNG BM -> YOUNG (N=14)

FIGURE 7. Bone marrow chimeric mice reconstituted with bone marrow (BM) from old donors display heightened liver metastases produced by intrasplenic injection of B16LS9 melanoma cell. (A) Old BM- > young recipients and (B) young BM - > young recipients and untreated young mice. (C) Untreated young mice used as a control group for comparison with young bone marrow chimera mice reconstituted with old bone marrow (old BM-> young). (D) Young mice that were either untreated (control) or were lethally irradiated and reconstituted with young bone marrow (young BM-> young). There were 20 mice in each bone marrow transplantation group and 14 mice in the control group.

murine tumor models there is a preferential expansion of granulocytic MDSCs.[60] Several studies have reported that MDSCs strongly suppress human and murine NK cytolytic activity.[60-62] Moreover, UM patients have increased numbers of granulocytic MDSCs in their peripheral blood.[63] These observations prompted us to examine the levels of granulocytic and monocytic MDSCs in the livers of old and young mice with liver metastases. Interestingly, there was a significantly lower percentage of granulocytic MDSCs ($CD11b^+Gr1^{high}$) in the livers of old mice, and there was an inverse situation with the monocytic MDSC ($CD11b^+Gr1^{low}$) population, which was significantly higher in the old mice (Fig. 8).

DISCUSSION

Immunosenescence is the condition in which adaptive and innate immune responses degenerate with age and is closely correlated with the development of cancer. The annual incidence of cancer per 100,000 people increases from 9.1 in individuals younger than 50 years to more than 400 in people older than 65 years.[64] The mean age of diagnosis of UM is 60.4 years, and the incidence increases progressively with age until the age of 70.[65]

The present study used a model of intraocular melanoma in which tumor cells are injected into the vitreous cavity as a means of producing melanomas that invade the uveal tract and metastasize to the liver, thereby recapitulating the natural
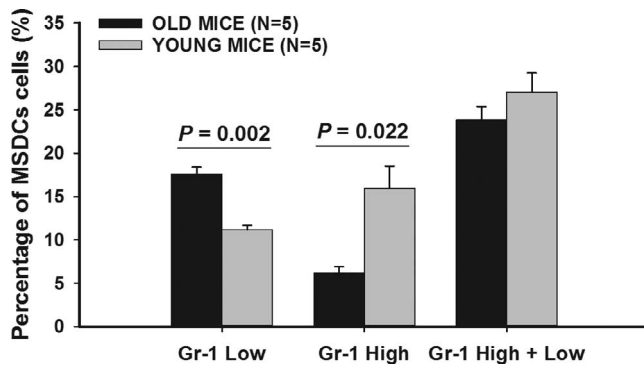
FIGURE 8. Murine CD11b$^+$Gr1$^+$ MDSC populations in livers of old and young mice following intrasplenic injection of B16LS9 melanoma cells. Murine CD11b$^+$ MDSCs were isolated based on their expression of the Gr1epitope. The Gr1high + low group was a combination of both high and low Gr1-expressing CD11b$^+$ MDSCs. This experiment was performed three times with similar results. There were five mice/group/experiment.

history of human UM. Unexpectedly, we found that melanomas grew slower in the vitreous cavity of old mice compared with young mice. The nature of this intraocular tumor model required us to enucleate the tumor-bearing eyes of young mice at day 14 to prevent the tumor from rupturing the globes. By contrast, the intraocular tumors in old mice did not reach a size indicating that rupture of the globe was imminent by day 14. Accordingly, we enucleated the tumor-bearing eyes of young and old mice at day 14 to allow comparison of tumor volume in both groups of mice at the same time point. The measurements of tumor volume by two independent methods consistently revealed that tumors grew slower in the PC in eyes of old mice, even when ocular macrophages were depleted by subconjunctival injection of clodronate-containing liposomes. These findings are in sharp contrast to a previous study, which reported that depletion of intraocular macrophages produced a steep reduction in the growth of AC melanomas in old but not young mice.[52] A number of explanations come to mind to account for this discordance in results. In the present study, we defined old mice as being older than 18 months, whereas the previous study by Ly et al.[52] categorized old mice as 10 to 12 months old. In our study, melanoma cells were introduced into the vitreous cavity as opposed to AC tumor injections used by Ly et al.[52] In spite of the differences in these two models, a number of important conclusions can be made from the present findings.

The slower growth of tumors in the eyes of aged mice seems counterintuitive, especially in the face of the diminished immune responses that occur with aging. However, the immune privilege of both the anterior and posterior regions of the eye would create an immunosuppressed environment for intraocular tumors in both young and old mice and would put tumor immune responses on an even footing for both categories of mice. The slower growth of tumors in the eyes of aged mice could be traced to multiple conditions, including differences in tumor angiogenesis and local growth factors within the eye, but the likelihood that disparities in either the adaptive or innate immune responses in the eyes of aged mice compared with young mice is highly unlikely based on the results shown in Figure 2, which indicated that disabling either the adaptive or innate arm of the immune responses did not affect the tempo of intraocular tumor growth.

Tumor size, as defined by thickness and largest tumor dimension, is a time-honored predictor for UM malignancy and the development of metastases.[66,67] However, multiple parameters affect the malignancy of UM, including, but not restricted to, the lesion size and thickness, morphology of the melanoma cells, gene expression profiles, and location of the primary lesion.[65,67-69] The results reported here suggest that mere tumor volume alone does determine the severity of liver metastases arising from intraocular melanomas and that aging has a profound effect on the development of liver metastases that is independent of the size of the intraocular tumor. The increased metastasis in old mice correlates with a depression in both the number and cytolytic activity of NK cells in the liver, even in tumor-free, control mice. The latter finding is consistent with reports in other tumor models in which aging is associated with diminished NK and NKT cell activity.[2] The increased incidence of liver metastases in old mice could be recapitulated by reconstituting lethally irradiated young mice with bone marrow from old mouse donors. In these mice, any "young" NK cells that might have escaped deletion by lethal whole-body irradiation would have been replaced by NK cells derived from bone marrow cells from the old mouse donors, as these mice were not used for 8 weeks. This is more than a sufficient amount of time for the original NK cells in young mice to die off and be replaced, as the half-life of NK cells in the mouse is 17 days or less.[56,57] The bone marrow reconstitution experiments clearly revealed that the increased incidence of melanoma metastases in old mice could be traced to bone marrow–derived cells, presumably NK cells, and was not directly attributable to parenchymal cells in the liver.

There is a growing awareness that MDSCs suppress both innate and adaptive tumor immune responses. In particular, granulocytic MDSCs (Gr1^{high} CD11b$^+$) are associated with tumor progression in several murine models.[60] Importantly, MDSCs strongly suppress human and murine NK cytolytic activity.[60-62] Moreover, UM patients have increased numbers of granulocytic MDSCs in their peripheral blood.[63] However, the present results revealing a significant decrease in granulocytic MDSCs in the livers of old mice suggests that MDSCs do not contribute to the depressed liver NK activity that occurs in old mice harboring liver metastases.

Collectively, our results confirm what one might expect to occur in UM. That is, advancing age leads to a degradation of the NK cell repertoire in the liver, the organ that has the highest concentration of NK cells and that coincidentally is the most common location for metastases arising from intraocular melanomas. This in turn leads to a steep increase in the development of melanoma liver metastases with advancing age. However, the delayed growth of intraocular tumors in aged mice was unexpected, and suggests that aging affects the growth kinetics of intraocular melanomas, yet the delayed growth does not translate to a commensurate reduction in metastases. Thus, aging has remarkably different effects on primary intraocular tumors and their metastases.

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