ORIGINAL RESEARCH

Contribution of Extra-Cardiac Cells in Murine Heart Valves is Age-Dependent

Lindsey J. Anstine, PhD; Tori E. Horne, BS; Edwin M. Horwitz, MD, PhD; Joy Lincoln, PhD

***Background*—**Heart valves are dynamic structures that open and close over 100 000 times a day to maintain unidirectional blood flow during the cardiac cycle. Function is largely achieved by highly organized layers of extracellular matrix that provide the necessary biomechanical properties. Homeostasis of valve extracellular matrix is mediated by valve endothelial and interstitial cell populations, and although the embryonic origins of these cells are known, it is not clear how they are maintained after birth. The goal of this study is to examine the contribution of extracardiac cells to the aortic valve structure with aging using lineage tracing and bone marrow transplantation approaches.

***Methods and Results*—**Immunohistochemistry and fate mapping studies using *CD45-Cre* mice show that the contribution of hematopoietic-derived cells to heart valve structures begins during embryogenesis and increases with age. Short-term (6 weeks), CD45-derived cells maintain CD45 expression and the majority coexpress monocyte markers (CD11b), whereas coexpression with valve endothelial (CD31) and interstitial (Vimentin) cell markers were infrequent. Similar molecular phenotypes are observed in heart valves of irradiated donor mice following transplantation of whole bone marrow cells, and engraftment efficiency in this tissue is age-dependent.

***Conclusions*—**Findings from this study demonstrate that the percentage of CD45-positive extracardiac cells reside within endothelial and interstitial regions of heart valve structures increases with age. In addition, bone transplantation studies show that engraftment is dependent on the age of the donor and age of the tissue environment of the recipient. These studies create a foundation for further work defining the role of extracardiac cells in homeostatic and diseased heart valves. **(*J Am Heart Assoc*. 2017;6:e007097. DOI: 10.1161/JAHA.117.007097.)**

Key Words: bone marrow transplant • extra cardiac • heart valve • heart valve endothelial cell • heart valve interstitial cell

Heart valves are dynamic structures required to maintain unidirectional blood flow during the cardiac cycle. In healthy individuals, this is achieved by structure-function relationships between the cellular and extracellular components of the mature valve and the hemodynamic environment.[1] The biomechanical function of the valve is provided by highly organized layers of extracellular matrix (ECM) components that include collagen, proteoglycan, and elastin. Homeostasis of the ECM in adult valves is largely mediated by a population of valve interstitial cells (VICs) that reside within the core of the leaflets.[2] Once considered a homogeneous population of fibroblast-like cells, VICs are now being described as heterogeneous based on their differential molecular profiles and diverse embryonic origins.[3–5] A single layer of valve endothelial cells (VECs) covers the leaflet surface and encapsulates the VICs and ECM. Their function is to serve as a physical barrier against the hemodynamic environment and molecularly communicate with underlying VICs to regulate their behavior.[6–8] The valve leaflets must open and close over 100 000 times a day, but despite the high mechanical demand, turnover of the VEC and VIC populations in adult murine valves is limited to ≈2.0% and 1.1%, respectively.[6] However, mouse models targeting functional alterations in either of these cell populations leads to aberrations in ECM homeostasis and associated mechanical failure.[9] Therefore, interest has grown in understanding how valve cell populations are maintained in adult valves to preserve structure-function relationships throughout life.

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Clinical Perspective

What Is New?

- CD45-positive extracardiac cells reside in the endothelial and interstitial compartments of the adult heart valve.
- The percentage of CD45-positive extracardiac cells within heart valve structures increases with age.
- Bone marrow engraftment is dependent on the age of the donor and age of the tissue environment of the recipient.

What Are the Clinical Implications?

- Aberrations in the contribution of extracardiac cells to heart valve structures could underlie leaflet dysfunction.
- Maintaining or replenishing heart valve cell populations, or delivering therapeutic agents using extracardiac cells could be an effective treatment strategy.

The VIC population originates from several embryonic origins, and this is further diversified between semilunar (aortic, pulmonic) and atrioventricular (mitral, tricuspid) valve leaflets. The majority of VICs within the atrioventricular valves are derived from endothelial cells following endothelial-to-mesenchymal transformation beginning at embryonic day 9.5 in the mouse. During later stages of development, additional cell lineages give rise to the VIC-precursor population, including epicardial-derived cells that contribute to the parietal leaflets of the primitive mitral and tricuspid valves[10,11]; and neural crest cells that contribute to distal regions of the semilunar valves.[12,13] Together, these fate-mapping studies have identified several cell lineages as sources of the VIC population during embryonic development.

In adult valves, proliferation rates of resident VICs are low,[6] and therefore attention has been given in exploring mechanisms that contribute to maintaining the VIC population after birth. Studies have alluded to the reactivation of endothelial to mesenchymal transformation as a means of replenishing the VIC population in injured adult valves,[14,15] whereas others have demonstrated the presence of extracardiac cells in the endothelium of infarct hearts[16] and VIC populations of homeostatic valves.[17,18] However, the phenotype and function of extracardiac cells within the valve leaflets remains unclear.

Aging is a significant risk factor of heart valve insufficiency, affecting up to 13.2% of the population aged >75 years.[19] The late onset of valve disease is likely acquired and idiopathic, and similar to other age-related cardiovascular diseases associated with endothelial cell dysfunction.[6] In this current study, we sought to examine age-associated differences in the contribution of exogenous and transplanted extracardiac cells to the valve cell populations and determine their short-term molecular fate. We show that the percentage of CD45-positive cells within valve structures increases with age. Additionally, cells from this lineage maintain CD45 identity, and largely coexpress markers for circulating monocytes, whereas coexpression with valve cell lineage marker was infrequent. In addition, bone marrow transplantation (BMT) experiments show that the fate of donor cells in heart valve structures is dependent on the age of both the donor and tissue environment of the recipient. These studies, for the first time, describe the contribution of exogenous and transplanted extracardiac cells to aging heart valve structures and identify their limited capacity to express molecular markers of resident valve cell populations.

Methods

Mice

Wild-type (WT) *C57BL/6J* and *C57BL/6-Tg(CAG-EGFP)1Osb/J (eGFP)* were obtained from The Jackson Laboratory (Bar Harbor, ME), and *CD45-Cre* and *Ai9(RCL-tdT)* reporter mice were a kind gift from Dr Edwin Horwitz at The Research Institute at Nationwide Children's Hospital (Columbus, OH). Male *CD45-Cre* mice were crossed with female *Ai9* mice to report Cre recombinase activity in embryonic day 11.5, postnatal day (PND) 2, and 6-week-old adult *CD45-Cre*$^{+}$*;Ai9*$^{+/-}$ progeny. *CD45-Cre*$^{-}$*;Ai9*$^{+/-}$ littermates were used as controls. All animal procedures were approved and performed in accordance with Institutional Animal Care and Use Committee and institutional guidelines provided by The Research Institute at Nationwide Children's Hospital.

Histology

Whole embryos, hearts, and livers from embryonic, postnatal day 2, and adult mice were dissected and fixed overnight in 4% PFA/1× PBS at 4°C and subsequently processed for paraffin or cryo embedding. Adult mice underwent whole-body perfusion with 1× PBS before dissection and heart tissue fixation. For paraffin sections, 7-μm sections were cut and subjected to immunofluorescent (IF) staining. Briefly, after deparaffinization, slides underwent antigen retrieval (H-3300; Vector Laboratories, Burlingame, CA) according to the manufacturer's protocol. Sections were blocked for 1 hour at room temperature (1% BSA, 0.1% cold water fish skin gelatin, 0.1% Tween 20/1× PBS, and 0.05% NaN_3), followed by incubation with primary antibody diluted in 1:1 Block/1× PBS overnight (see Table for antibodies and concentrations). 24 hours later, slides were incubated in Alexa Fluor secondary antibodies diluted at 1:400 in 1× PBS for 1 hour at room temperature, mounted in Vectashield containing DAPI, and imaged on an Olympus BX51 microscope (Olympus Corporation, Tokyo, Japan). Alternatively, hearts and livers were processed and embedded for cryo and cut at

Table. Antibodies and Working Concentrations

Primary Antibody	Vendor	Host	Dilution
GFP	Abcam (ab13970)	Chicken	1:500
GFP	Abcam (ab290)	Rabbit	1:1000
CD45	R&D Systems (AF114)	Rabbit	1:200
RFP	Rockland (600-401-379)	Rabbit	1:3000
Vimentin	Abcam (ab45939)	Rabbit	1:200
Vimentin	Novus Bio. (NB300-223)	Chicken	1:200
CD31	BD Biosciences (553370)	Rat	1:1000
F4/80	Bio Rad (MCA497GA)	Rat	1:200
CD11b	Bio Rad (MCA711G)	Rat	1:200
Cleaved caspase-3	Cell Signaling (9664)	Rabbit	1:200

GFP indicates green fluorescent protein; RFP, red fluorescent protein.

7-μm sections. Slides were then blocked for 1 hour at room temperature and stained as described above. Histological quantification was performed by counting the immunoreactive cells of interest and total DAPI$^+$ cells in every 18th tissue section spanning the aortic or mitral valve region of adult mice, every ninth section for postnatal, and every sixth section for embryonic (n=3). Results are reported at a percentage of total cells. Significance was found using the Student *t* test between comparative time points or experimental groups.

Bone Marrow Transplants

Preparation of donor cells

Femur and tibia bones from 7-week-old or >12-month-old *eGFP* (enhanced green fluorescent protein) female donors were collected, rinsed in 1× HBSS containing 1% penicillin/streptomycin, and kept on ice. Whole bone marrow cells were isolated by flushing the bone cavity with 5 mL of RPMI media containing 1% penicillin/streptomycin. Cells were strained through a 0.70-μm strainer and resuspended in sterile 1× HBSS at a concentration of 1.25×10^6 cells/mL.

Irradiation and BMTs

Seven-week-old and >12-month-old female *C57BL/6J* recipient mice received total body irradiation at 500 cG followed by a second 500 cG dose 3 hours later using an X-RAD 320 irradiator. 24 hours later, recipients received 250 000 whole bone marrow cells collected from either 7-week-old or >12-month-old *eGFP* donors by tail vein injection. At 11 weeks post-BMT, recipient mice were euthanized and subjected to whole-body gravity perfusion with 1× PBS. Organs, including the heart and liver, were collected and fixed overnight in 4% PFA.

Peripheral blood reconstitution analysis

Peripheral blood reconstitution levels were determined at 3 weeks post-BMT. Briefly, recipient mice underwent submandibular bleeding. Blood was incubated in 1× red blood cell lysis buffer (BioLegend, San Diego, CA) and kept 15 minutes in the dark. Afterward, samples were washed, resuspended in 1× HBSS containing 10% FBS, and submitted for flow cytometric analysis for the presence of green fluorescent protein (GFP). The number of GFP$^+$ cells was reported as a percentage of total cells within the blood. Blood from homozygous *eGFP* mice and WT *C57BL/6J* were used as positive and negative controls, respectively.

Liposomal clodronate injections into BMT mice

BMTs in 7-week-old female *C57BL/6J* recipients were carried out with whole bone marrow from 7-week-old or >12-month-old *eGFP* donors (see above). At 7 weeks post-BMT, recipients received intraperitoneal injections of either control empty liposomes or liposomes containing Clodronate (#8901; Encapsula NanoSciences, Nashville, TN). Mice were injected every 3 days for 30 days at 40-mg/kg body weight for the first dose and 20-mg/kg body weight for all subsequent injections.[20] At 11 weeks post-BMT, recipient mice were euthanized and subjected to whole-body gravity perfusion with PBS. Organs, including the heart, were collected and fixed overnight in 4% PFA.

To assess the macrophage depletion efficiency of Clodronate-treated mice, splenocytes and peripheral blood were isolated from mice injected with control empty liposomes and liposomes containing Clodronate at 11 weeks post-BMT. Red blood cells were lysed with 1× red blood cell lysis buffer and samples were blocked in Mouse BD FC Block (#553141; BD Biosciences, San Jose, CA). After blocking, cells were stained for F4/80 (#123116, 1:200; BioLegend), GR1 (#108433, 1:400; BioLegend), Cd11b (#101208, 1:400; BioLegend) diluted in PBS containing 0.1% sodium azide and 1% BSA for 20 minutes on ice (an isotype control against F4/80 was also prepared [#400512; BioLegend]). After staining, cells were washed and resuspended in Near IR Live/Dead Cell Stain Kit (Life Technologies, Carlsbad, CA) for 30 minutes at room temperature. Subsequently, cells were fixed in 1% PFA for 15 minutes at room temperature and submitted for flow analysis to determine the number of cells expressing each marker of interest. Data were analyzed using FlowJo software (Tree Star, Inc, Ashland, OR).

Cell Cycle Analysis

PND2 *CD45-Cre*$^+$*;Ai9*$^{+/-}$ mice were injected subcutaneously with 5 μg/g body weight of EdU (Invitrogen, Carlsbad, CA). Twenty-four hours later, mice were euthanized and hearts

were harvested. Tissue was fixed in 4% PFA overnight and processed for cryo embedding. 7μm sections were subjected to Click-it EdU (Invitrogen) detection and IF staining for red fluorescent protein (RFP; 600-401-379 1:3000; Rockland Immunochemicals Inc, Pottstown, PA), according the manufacturer's instructions. Proliferating *CD45-Cre*$^{+}$;*Ai9*$^{+/-}$ cells were detected by coexpression of RFP and EdU. Every 9th tissue section spanning the aortic and mitral valve region was collected and used for quantification (n=3). Results are reported as a percentage of total *CD45-Cre*$^{+}$ cells.

Statistical Analysis

For all statistical analysis, experimental values were based on the average of at least 3 biological replicates, and a paired Student *t* test was performed and significance was determined as $P<0.05$ as indicated in figure legends.

Results

The Percentage of CD45-Positive Cells in Heart Valves Increases With Age and Maintains CD45 Expression

Previous studies have described the presence of bone-marrow–derived cells in adult heart valves,[17,18] but few have looked at endogenous levels of the hematopoietic marker, CD45, over time. Here, we used *C57/Bl6* WT mice to show that CD45-positive cells are detected in endocardial cushions of primitive aortic valves at embryonic day 14.5, and reactivity is also observed in the mitral position, but the percentage of cells is significantly lower (Figure 1A). It is not known whether these cells are embryonic hematopoietic stem cells derived from primitive or definitive hematopoiesis, or tissue-resident hematopoietic cells.[21] Similar percentages of CD45-positive cells are observed at postnatal stages in both valve sets (≈1.2% aortic, ≈2.6% mitral), but the percent significantly increases by 4 months (≈8.0% aortic, ≈6.7% mitral) and 16 months (≈10.9% aortic, ≈10.5% mitral) of age (Figure 1A). Therefore, the contribution of CD45-positive cells to heart valve structures increases with age.

To support endogenous CD45 studies in heart valves (Figure 1A), we examined the contribution and fate of these cells to heart valve structures using the *CD45-Cre* model[20] bred in with *Ai9(RCL-tdT)* reporter mice[22] to permanently label cells derived from the CD45 lineage with red fluorescence. As shown in Figure 1B, the percent contribution of CD45-derived cells is low in the primitive valve structures at embryonic day 11.5, similar to endogenous expression, and the percent of *CD45-Cre;Ai9-RFP*-positive cells increases with age (≈2.3% at postnatal stages, and ≈10.3% at 6 weeks). To determine the fate of these cells in vivo, colabeling of RFP (to detect *CD45-Cre;Ai9-RFP* transgene) was performed with markers of the myeloid and valve cell lineages. This was examined at 6 weeks of age because the percentage of CD45-positive cells at this time was similar to aging mice (Figure 1A). Figure 1C and 1I shows that the majority (≈95.1%) of cells derived from the CD45-lineage maintain endogenous CD45 expression (arrowhead Figure 1C), suggesting insignificant changes in cell fate and persistence of the myeloid phenotype. To further explore this, RFP was costained with markers of the myeloid lineage in aortic valves of 6-week-old *CD45-Cre;Ai9-RFP* mice, including CD11b to detect circulating monocytes and F4/80, a marker of mature macrophages. Using IF we report that ≈65% of CD45-derived (RFP-positive) cells costained with CD11b (arrowhead, Figure 1D and 1I) and only ≈4% were RFP and F4/80 double positive (Figure 1E and 1I). Figure 1F serves as a positive control and shows F4/80 reactivity in the adult liver following radiation-induced injury. Of note, we did not detect any CD11b- or F4/80-positive cells in the valves that were not derived from the CD45 lineage.

To determine whether CD45-derived cells contribute to the resident valve cell populations, cells were double stained with RFP and CD31 to detect valve endothelial cells, and Vimentin, a marker highly enriched in both valve interstitial and endothelial cells.[3] By IF, colocalization of RFP with these markers was comparatively low (≈16% CD31, ≈11% Vimentin; Figure 1G and 1H), and based on the high levels (≈93%) of overlap of RFP with CD45, it is anticipated that these CD45-derived valve cells also express endogenous CD45. Worthy of mention, we did not identify a significant percentage of CD45-derived cells undergoing proliferation as determined by EdU incorporation (data not shown). Together, these descriptive studies support Figure 1A and confirm that the contribution of CD45-positive cells to heart valve structures increases with age. In addition, our fate mapping approach suggests that CD45-derived cells maintain CD45 expression and myeloid phenotypes and do not significantly contribute to VIC or VEC populations at the molecular level.

Distribution of Transplanted Bone Marrow Cells Within Endothelial and Interstitial Compartments of the Mature Heart Valve Is Affected by Age

Our findings suggest that circulating CD45-positive cells contribute to heart valve structures and the percentage is age-dependent. To determine whether circulating, extracardiac bone marrow cells similarly home to adult valves, transplantation experiments were performed using young (7 week) and aging (>12 month) donors and recipients to further examine the effects of aging on this process. As shown in Figure 2A, 7-week-old (young) and >12-month-old (aging) *C57/Bl6* WT

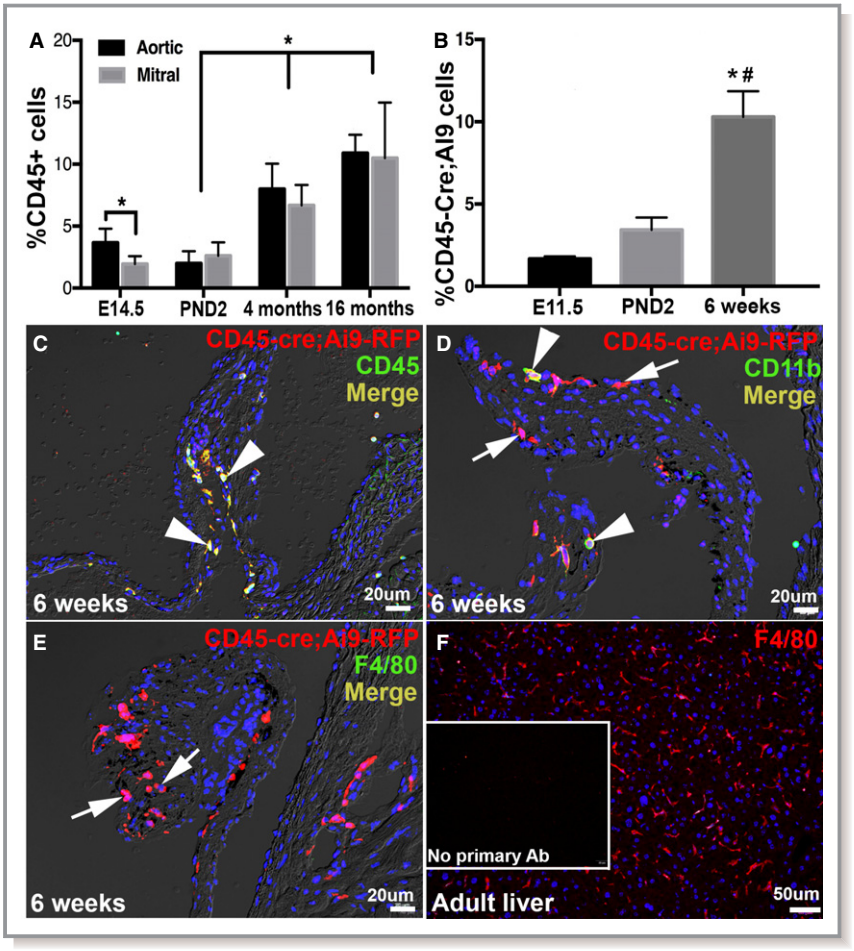

Figure 1. In C57/Bl6 wild-type mice, the percentage of CD45$^+$ cells in aortic and mitral valve leaflets increases with age and maintain CD45 expression. A, CD45$^+$ cells represented as a percentage of total cells at each time point in aortic and mitral valves. B, Percentage of CD45-derived (RFP$^+$) cells in aortic valves from *CD45-Cre$^+$;Ai9$^{+/-}$* mice at E11.5, PND2, and 6 weeks of age (*P<0.05 compared with E11.5; $^{\#}P$<0.05 compared with PND2). C, Aortic valve from 6-week-old *CD45-Cre$^+$;Ai9$^{+/-}$* mice, stained for RFP (red) to detect CD45-derived cells, and for CD45 (green) to detect endogenous CD45 expression. D and E, 6-week-old *CD45-Cre$^+$;Ai9$^{+/-}$* aortic valves costained with RFP (red) and immune (CD11b [D] and F4/80) [E]) (green) cell markers. F, F4/80 immunoreactivity in adult liver following irradiation-induced injury. Negative control of no primary antibody (Ab) is shown as inset. G and H, 6-week-old *CD45Cre$^+$;Ai9$^{+/-}$* aortic valves costained with RFP (red) and CD31 (endothelial) (G) and Vimentin (interstitial) (H) (green). Arrowheads: double-positive cells. Arrows: CD45-derived cells negative for CD11b, F4/80, CD31, and Vimentin. Notched arrowheads: CD11b-, F4/80-, CD31-, or Vimentin-positive cells that are not CD45 positive. I, Quantification of the number of CD45-derived cells expressing endogenous CD45, CD11b, F4/80, CD31, and Vimentin. n=3. Statistical significance based on *P<0.05 between each group. E indicates embryonic day; PND, postnatal day; RFP, red fluorescent protein.

recipient mice were subject to total-body lethal irradiation following exposure to 2, 500 cG treatments. 24 hours later, recipients received a single bolus of 250 000 whole bone marrow cells isolated from 7-week-old (young) or >12-month-old (aging) *eGFP* donor mice. Flow cytometry analysis determined that blood reconstitution 3 weeks following transplantation of bone marrow cells from 7-week-old *eGFP* donors was ≈61% in 7-week-old recipients and ≈69% in >12-month-old recipients. In contrast, reconstitution in 7-week recipients receiving bone marrow from >12-month-old *eGFP* donors was ≈51% and ≈32% in >12-month recipients (data not shown). This suggests that the reconstitution potential is largely dependent on the age of the donor bone marrow cells and, to a lesser extent, the age of the recipient.

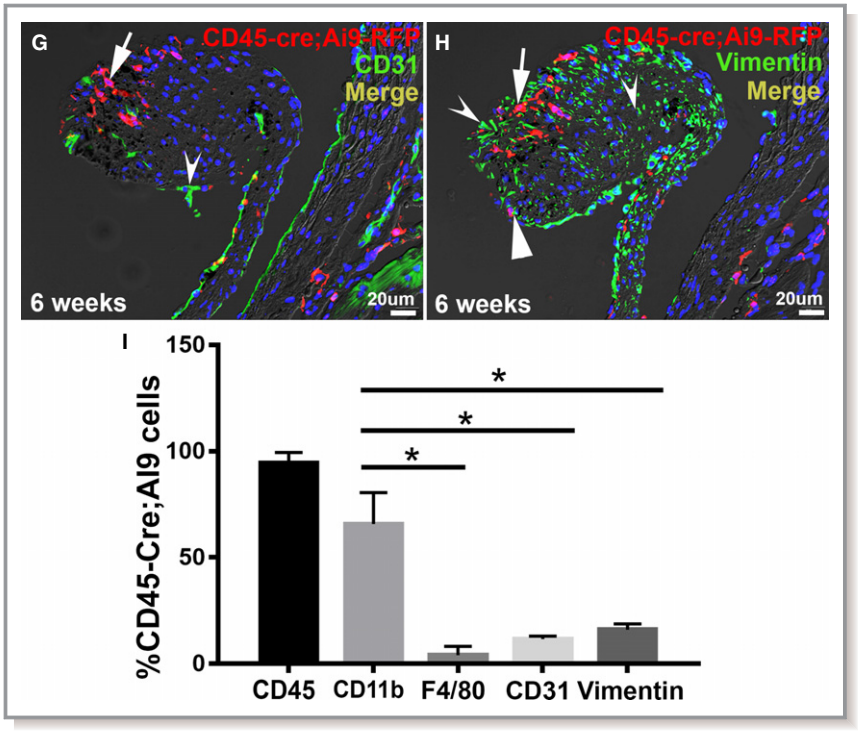

Figure 1. Continued

At 11 weeks post-BMT, hearts from recipient mice were harvested and analyzed for detection of GFP-positive cells within heart valve structures. In studies utilizing the bone marrow cells harvested from the 7-week-old *eGFP* donors, we observed ≈6% GFP-positive cells within the aortic valve of 7-week-old recipients and a significantly higher percent (≈8%) in >12-month-old recipients (Figure 2B through 2D). To confirm that homing of GFP-positive cells to the valves was not the result of injury following irradiation, cleaved Caspase-3 immunoreactivity was examined 24 hours following exposure to 2, 500 cG treatments. In data not shown, few apoptotic cells were detected by this marker and no significant differences were observed between nonirradiated age-matched WT control mice. To further examine the contribution of transplanted bone marrow cells to the aortic valve structure, the distribution of *eGFP* cells was determined as a percent of total cell number within the endothelium (costained with CD31) and interstitial space (CD31-negative within the valve cusp). In 7-week-old donor studies, the distribution of *eGFP* cells in 7-week-old recipients was comparable between the endothelium and interstitium (≈6% each; Figure 2B through 2D). However, the majority of 7-week-old *eGFP* donor cells into the >12-month-old recipient were enriched within the core of the valve cusp, particularly the ventricularis region associated with the VIC population.

To determine whether the intrinsic age of the *eGFP* donor cells influences their contribution and distribution into aortic valve structures of 7-week-old and >12-month-old recipients, the BMT procedure was repeated using >12-month-old *eGFP* donors. As indicated in Figure 2E through 2G, the percentage of *eGFP* cells in the aortic valve was significantly higher (≈7%) in 7-week-old, over >12-month-old (≈2%), recipients. Furthermore, this ≈2% contribution in >12-month-old recipients is significantly less than observations made in the same age mice following transplantation with *eGFP* cells from 7-week-old donors (comparing Figure 2D with 2G). This suggests that bone marrow cells from >12-month-old donors have decreased engraftment into >12-month-old, but not 7-week-old, recipient heart valves.

Our analysis further shows that *eGFP* cells from aging donors favors localization in the interstitium compartment over the endothelium in both 7-week-old (Figure 2E and 2G) and >12-month-old (Figure 2F and 2G) recipients. This is in contrast to the 7-week donor study that showed equal distribution of *eGFP* cells from 7-week-old recipients in both compartments (Figure 2D) and a significantly higher contribution in the endothelium compared with >12-month-old studies (compare Figure 2D through 2G). As a percentage, this number of *eGFP* cells from >12-month-old donors within the interstitium was significantly higher in 7-week-old recipients, whereas this observation was opposite in 7-week donors. Taken together, these transplantation studies show that 7-week-old (young) recipients engraft a similarly high percentage of transplanted bone marrow cells from young and aging (>12-month) donors. However, significantly less transplanted cells are observed in valves following transplantation

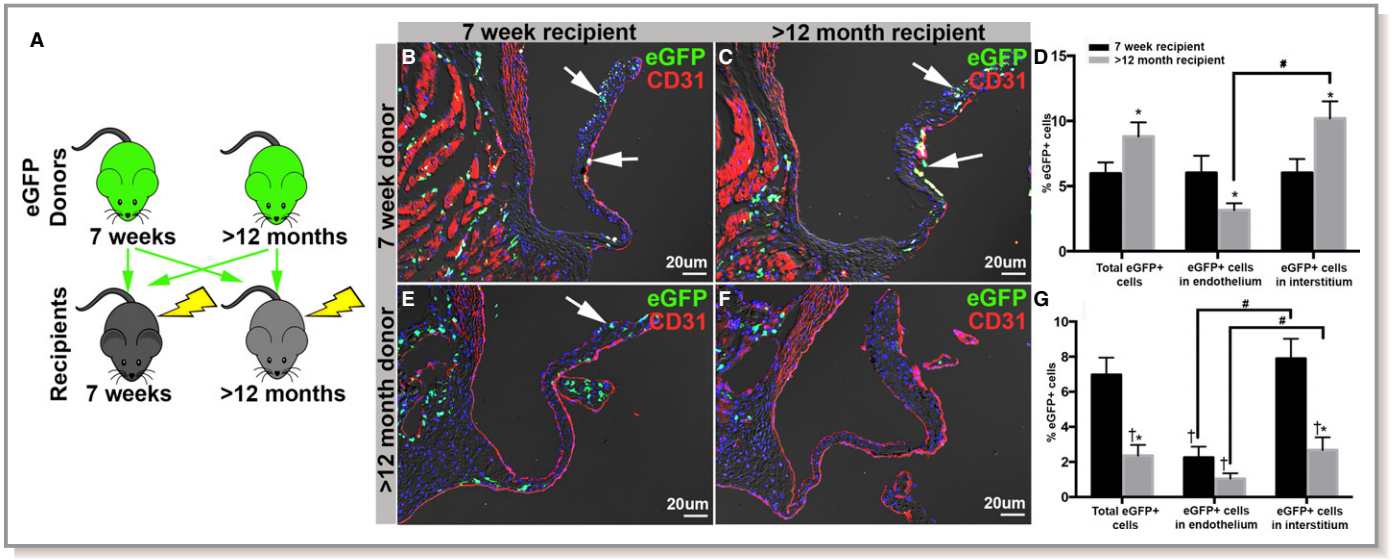

Figure 2. Contribution of transplanted bone-marrow–derived cells to the aortic valve is an age-dependent process. A, Schematic diagram of bone marrow transplants using 7-week-old and >12-month-old eGFP donor mice and 7-week-old and >12-month-old wild-type recipient mice. B through F, Immunofluorescent analysis for eGFP (green) and CD31 (red) in aortic valves from 7-week-old (B and E) and >12-month-old (C and F) BMT recipients reconstituted with bone marrow from a 7-week-old donor (B and C) or >12-month-old donor (E and F) to show integration of $eGFP^+$ cells into the VIC and VEC (arrows) compartments. D and G, Percentage of $eGFP^+$ cells, $eGFP^+$ $CD31^+$ double-positive (VEC compartment) and $eGFP^+$ CD31-negative (VIC compartment) cells in BMT recipients transplanted with 7-week-old bone marrow (D) or >12-month-old bone marrow (G). n=3. **P*>0.05 in 7 week recipient compared to >12 month recipient $^{\#}P$>0.05 in eGFP+ cells in interstitium compared to eGFP+ cells in endothelium. BMT indicates bone marrow transplant; eGFP, enhanced green fluorescent protein; VEC, valve endothelial; VIC, valve interstitial cell.

of donor cells from aging mice into aging recipients. Furthermore, we conclude that young donor cells are equally distributed in the endothelium and interstitial compartments in young recipients, but favor the interstitium in aging recipients. In contrast, aging donor cells similarly home into the interstitium in young and aging recipients. Therefore, the age of the transplanted bone marrow cells and the age of the recipient influence the homing of extracardiac cells to the aortic valve.

Age of the Valvular Tissue Environment Influences the Phenotypic Heterogeneity of Transplanted Bone Marrow Cells Located Within the Valve Leaflets

To determine the fate of *eGFP* cells from 7-week-old (young) donors in aortic valves of young and aging (>12-month) recipient mice (Figure 3A), we examined colocalization of GFP with myeloid and valve cell population markers as described in Figure 1. As observed in *CD45-Cre;Ai9-RFP* mice, the majority (>98%) of *eGFP* donor cells from 7-week or >12-month mice maintained expression of CD45 (data not shown). In addition, the majority (≈46.2%) of *eGFP* transplanted cells from 7-week-old donors costained for both GFP and CD11b (Figure 3B and 3J). Consistent with *CD45-Cre;Ai9-RFP* mice, significantly less *eGFP* donor cells from 7-week-old donors were positive for F4/80 (≈5.6%; Figure 3C, 3G, and 3J), CD31 (≈8.1%; Figure 3D, 3H, and 3J), or Vimentin (≈8.0%; Figure 3E, 3I, and 3J) in 7-week-old recipients when compared with CD11b cell numbers. The molecular phenotype of 7-week-old *eGFP* donor cells was different in >12-month-old recipients compared with younger recipients, including fewer cells coexpressing GFP with CD11b (≈10.4%; Figure 3F and 3J), F4/80 (≈1.3%; Figure 3G and 3J), CD31 (≈2.7%; Figure 3H and 3J), and Vimentin (≈13%; Figure 3I and 3J). In addition, young donor cells in aging recipients show enrichment of cells coexpressing GFP with Vimentin, suggesting that transplanted bone marrow cells from young donors have greater myeloid potential in younger recipients, and the molecular profile of young transplanted cells is similar to the native CD45 lineage.

To complement studies utilizing young *eGFP* bone marrow cell donors, experiments were repeated to determine the fate of *eGFP* cells from aging (>12 months) donors in young and old recipients. Similar to young donors, aged *eGFP* bone marrow cells were >98% positive for CD45 (data not shown); however, significantly fewer aging *eGFP* donor cells costained with CD11b (Figure 4J) when compared with young donors (Figure 3J). In addition, unlike findings using bone marrow cells from young donor mice, there were no significant differences in the percentage of GFP and CD11b double-positive cells in young and aging recipients receiving bone

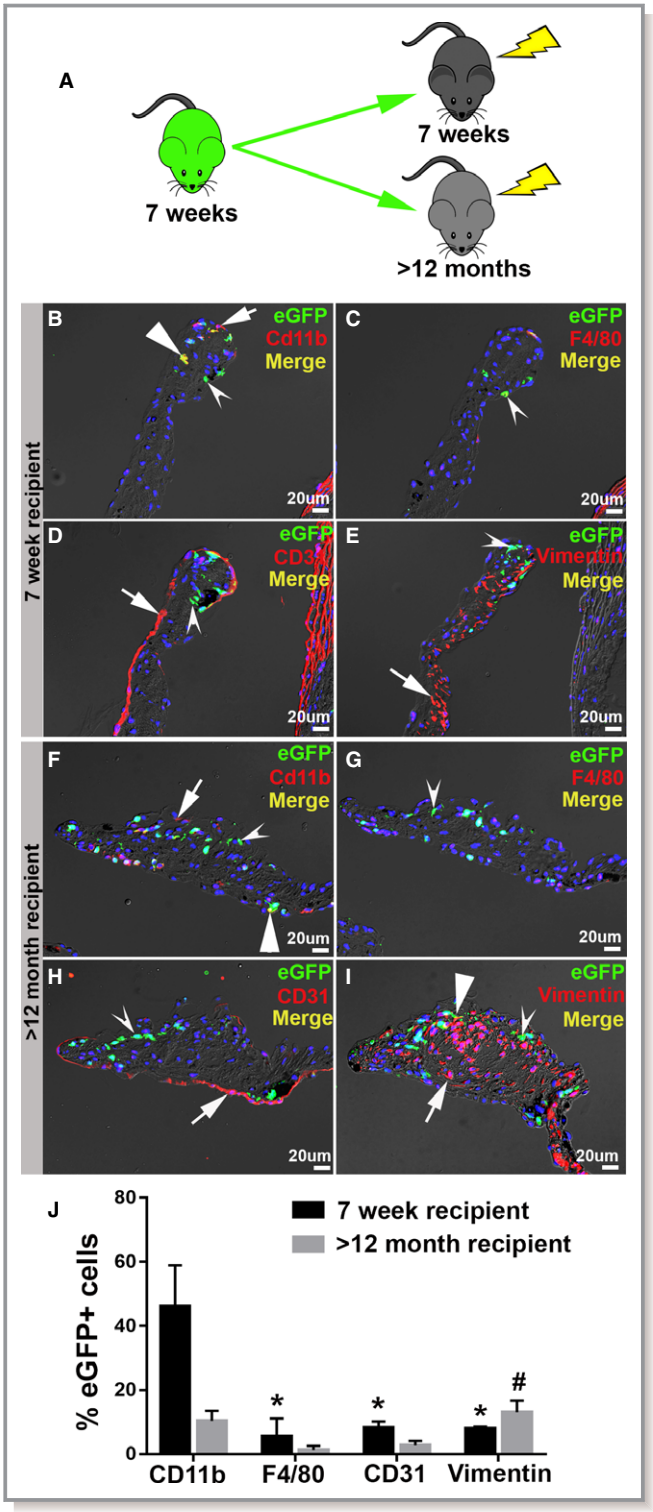

Figure 3. Heterogeneity of transplanted 7-week-old bone-marrow–derived cells in the aortic valve is affected by the aging tissue environment. A, Schematic diagram to show BMTs using 7-week-old donors. B through I, Immunofluorescent staining of aortic valves from 7-week-old (B through E) and >12-month-old (F through I) BMT recipients who received bone marrow from a 7-week-old donor, against immune (CD11b and F4/80) (B, C, F, and G) and valve cell (CD31 and Vimentin) markers (green) (D, E, H, and I). Costaining with eGFP denotes transplanted cells in green. J, Percentage of $eGFP^+$ cells coexpressing CD11b, CD31, Vimentin, and F4/80 in 7-week-old recipients and >12-month-old recipients. Arrowheads: double-positive cells. Arrows: CD11b-, F4/80-, CD31-, or Vimentin-positive cells that are negative for eGFP. Notched arrowheads: bone-marrow–derived ($eGFP^+$) cells negative for CD11b, F4/80, CD31, or Vimentin expression. n=3. Statistical significance based on $P<0.05$ compared to $CD11b^+$ cells. * $P>0.05$ compared to CD11b+ cells # $P>0.05$ compared to 7 week recipient BMT indicates bone marrow transplant; eGFP, enhanced green fluorescent protein.

marrow cells from aging donors (Figure 4B, 4F, and 4J). This pattern of insignificant differences between young and aging recipient mice following transplantation with aging *eGFP* donor cells was also true for F4/80 (Figure 4C, 4G, and 4J), CD31 (Figure 4D, 4H, and 4J), and Vimentin (Figure 4E, 4I, and 4J). Although only few *eGFP* donor cells from young mice coexpressed F4/80 in young and aging recipient (Figure 3J), coexpression was not detected in donor cells from aging mice (Figure 4J). However, aging donor cells did coexpress CD31 and Vimentin at levels similar to younger *eGFP* bone marrow cells when transplanted into young recipients. However, CD31/*eGFP* coexpression was undetected in aging donor cells transplanted into aging recipients (Figure 4J). These findings indicate that transplanted bone marrow cells from aging mice have lower potential toward myeloid and valve cell phenotypes and this is independent of age of the recipient.

Transplanted Bone Marrow Cells in Homeostatic Heart Valves Do Not Favor Differentiation Towards a Mature Macrophage Phenotype

Macrophage infiltration has been well described in diseased or injured heart valves, yet our findings in this current study suggest that under homeostatic conditions, extracardiac cells express low levels of F4/80, and high levels of CD11b, suggesting a more-monocytic phenotype (Figures 1 through 4). To confirm that transplanted bone marrow cells from young and aging donors do not give rise to mature macrophages in aortic valves, 7-week-old recipient mice were irradiated and received *eGFP* cells from either young or aging donors. 7 weeks post-transplantation, mice were treated with 40 mg/kg of the macrophage-depletion drug, Clodronate, every 3 days for 30 days in order to deplete macrophages within adult murine tissues.[23] At 11 weeks post-transplant, macrophage depletion was assessed by flow cytometry to detect CD11b and F4/80 expression (Figure 5A). A 90.4% reduction in splenic macrophages in young recipients, and 95.7% reduction in aging recipients, was observed in

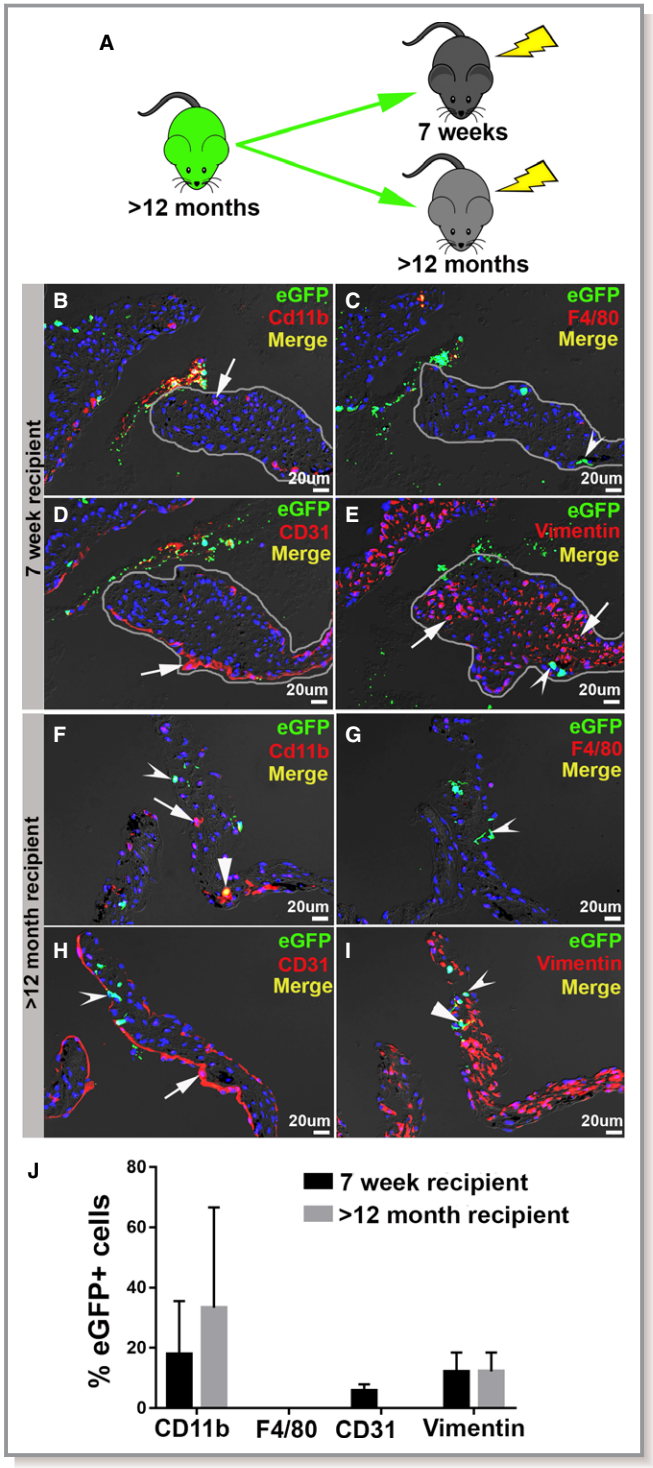

Figure 4. Transplanted bone-marrow–derived cells isolated from aged mice display altered molecular phenotypes that are influenced by the age of the native tissue environment. A, Schematic diagram to show BMTs using bone marrow cells isolated from >12-month-old donors. B through I, Immunofluorescent staining of aortic valves from 7-week-old (B through E) and >12-month-old (F through I) BMT recipients who received bone marrow cells from a >12-month-old donor, against immune (Cd11b and F4/80) (B, C, F, G) and valve cell (CD31 and Vimentin) markers (green) (D, E, H, and I). Costaining with eGFP denotes transplanted cells in green. J, Percentage of $eGFP^+$ cells expressing CD11b, CD31, Vimentin, or F4/80 in transplanted recipients (J). Arrowheads: double-positive cells. Arrows: CD11b-, F4/80-, CD31-, or Vimentin-positive cells that are negative for eGFP. Notched arrowheads: bone-marrow–derived ($eGFP^+$) cells negative for CD11b, F4/80, CD31, or Vimentin expression. n=3. Statistical significance based on $P<0.05$ compared to $CD11b^+$ cells. BMT indicates bone marrow transplant; eGFP, enhanced green fluorescent protein.

Clodronate-treated transplanted mice compared with vehicle control-treated transplanted mice, or WTs. Macrophage depletion was also observed in the liver of Clodronate-treated mice as determined by immunohistochemistry (data not shown). Using anti-GFP immunoreactivity, we examined the contribution of transplanted *eGFP* bone marrow cells in recipient mice that received bone marrow from 7-week-old (Figure 5B and 5C) or >12-month-old (Figure 5D and 5E) donor mice. As shown in Figure 5F, there was no significant difference in the percentage of GFP-positive cells in heart valves of Clodronate-treated mice compared with vehicle controls, despite successful macrophage depletion. Similar to our previous irradiation study (Figures 2 through 4), irradiation did not affect cell survivability in the valve because significant changes in apoptosis were not observed compared with nonirradiated controls (data not shown). This suggests that engrafted bone-marrow–derived cells in homeostatic aortic valves are largely not phenotypically mature macrophages.

Discussion

In 1998, a study by Koolbergen et al was the first to suggest that extracardiac cells contribute to heart valve structures after identifying both donor and recipient cells in patients receiving sex-mismatched cryo-preserved homograft valve transplants.[24] Since this early study, there have been few follow-up reports, and the native processes that mediate maintenance of valve cell populations in homeostasis and disease are still largely unknown. In this current study, we report an increasing percentage of CD45-derived cells within the valve structure from embryonic to adult stages and note their continued expression of CD45 and coexpression with CD11b, a marker of circulating monocytes. Interestingly, few CD45-derived cells express molecular markers common to VEC and VIC populations. In addition, we show that transplanted bone marrow cells are CD45 positive, suggesting hematopoietic origin, and home to endothelial and interstitial regions of the valve cusps. In 7-week-old (young) recipients receiving bone marrow from young donors, the majority of transplanted cells express CD11b (circulating monocytes).

However, this monocytic fate is less predominant when cells from young donors are transplanted into aging (>12 months) recipients, or aging donor cells are transplanted into young or aging recipients. These studies are the first to quantify changes in the contribution of extracardiac, CD45-positive cells to homeostatic valve structures during aging and to report the age-dependent changes in cell fate.

Previous studies have described the recruitment of bone-marrow–derived cells to the VIC population of homeostatic valve structures.[17,18] In contrast to our transplant studies using whole bone marrow cell preparations, Visconti et al isolated hematopoietic stem cells from donor mice and clonally expanded them in short-term culture before transplant. Using this approach, they reported the contribution of hematopoietic stem cell–derived clonal cells to the VIC, but not VEC population, based on location and expression of procollagen 1α1, 8 to 10 weeks following transplant,[17] and this observation was also observed following transplantation of bone marrow total nucleated cells.[25] In our current study, location studies are supported by immunohistological analysis to determine the molecular phenotype of extracardiac cells in the aortic valve based on a panel of markers representing the hematopoietic lineage (CD45), VECs (CD31), VICs (Vimentin), monocytes (CD11b), and mature macrophages (F4/80). Using these approaches, we observe ≈6% of CD45-derived and transplanted bone marrow cells coexpress CD31 within the endothelial lining 12 weeks after transplant. However, not all the extracardiac cells observed in the location of the endothelium (see Figure 2D and 2G) coexpress CD31, suggesting that, in our model, location alone is not sufficient to identify molecular phenotypes. In addition to the endothelial cell population, ≈11% and ≈8% of CD45-derived and transplanted bone marrow cells, respectively, express Vimentin, but the majority (≈26–65%) of extracardiac cells express CD11b. Worthy of mention, ≈93% of extracardiac cells in the valves express CD45 and therefore it is likely that additional expression of CD31, Vimentin, and CD11b is transient. However, additional long-term studies coupled with functional analysis will determine the true cell fate potential of these cells.

A novel observation from our study is the differential contribution and phenotypes of transplanted whole bone marrow cells from young and aging donors to the aortic valve structures in young and aging recipients (Figures 2 through 4). In young donor studies, we observed an overall increase in the percentage of transplanted cells engrafted into the valve and these cells preferentially locate to the interstitium, where they predominantly express CD11b (46.2%) in young recipients and Vimentin (13%) in aging recipients. In contrast, donor cells from aging mice showed no significant enrichment of molecular markers following transplantation into young recipients. Engraftment of aging donor cells into aging recipients was significantly less than other experimental groups and this, in part, could be explained by lower reconstitution rates. However, it may also reflect deterioration of the homing process in aging mice and defects in retention of extracardiac cells within the valve structures. It has been well described in other systems that tissue engraftment of bone marrow cells decreases with aging.[26–31] The homing process of extracardiac cells to the valves has not been examined, although a recent RNA-sequencing study by our lab showed that VECs express several chemokine ligands, including *CX3CL1*, that decreases with aging,[6] that might suggest that this cell population mediates the homing signal; however, further work is required to determine this. In this study, we show localization of transplanted GFP-positive cells within the endothelium 11 weeks after transplant. The long-term fate of these cells in the endothelial compartment has yet to be determined, but it is considered that VEC-mediated homing signals attract circulating extracardiac cells, leading to adhesion to the endothelium and transient CD31 expression and, over time, infiltration into the interstitium.[6] In the context of aging, we have shown that VECs generate excess reactive oxygen species and accompanying changes in mitochondrial metabolism[6] and these may contribute to defects in the homing process. In addition, aging is associated with epigenetic changes within cells, including DNA methylation, that could lead to repression of homing signals on the surface of the aortic valve leaflet.[32] Taken together, our studies have shown that engraftment of extracardiac cells to the aortic valves is dependent on the age of both donor bone marrow cells and tissue microenvironment.

Our study focused on the recruitment of extracardiac circulating cells in homeostatic aortic valves, and, under these conditions, we observed a baseline percent of CD45-positive cells that increased with age. We rarely detected proliferation in this population, and therefore observed increases are likely attributed to continued infiltration throughout life. The most significant increase in endogenous CD45 expression was observed between postnatal day 2 and 4 months of age. The role of extracardiac cells during this stage in the absence of disease is intriguing and points to a role in physiological growth and remodeling of the valve structure. Further increases in CD45 expression were also observed between 4 and 16 months of age, and this could be attributed to an adaptive response attributed to age-related deterioration of the valve structure, including compromised cell function and degradation of the ECM.[6,33] Additional work is needed to determine the role of CD45-positive cells in the homeostatic valve. Our data suggest that, short term, these cells are not phenotypic of resident valve cells under these conditions, but closely resemble immature monocyte-type cells. Therefore, it is speculated that in the absence of disease, this CD45-positive, CD11b-enriched population remains functionally

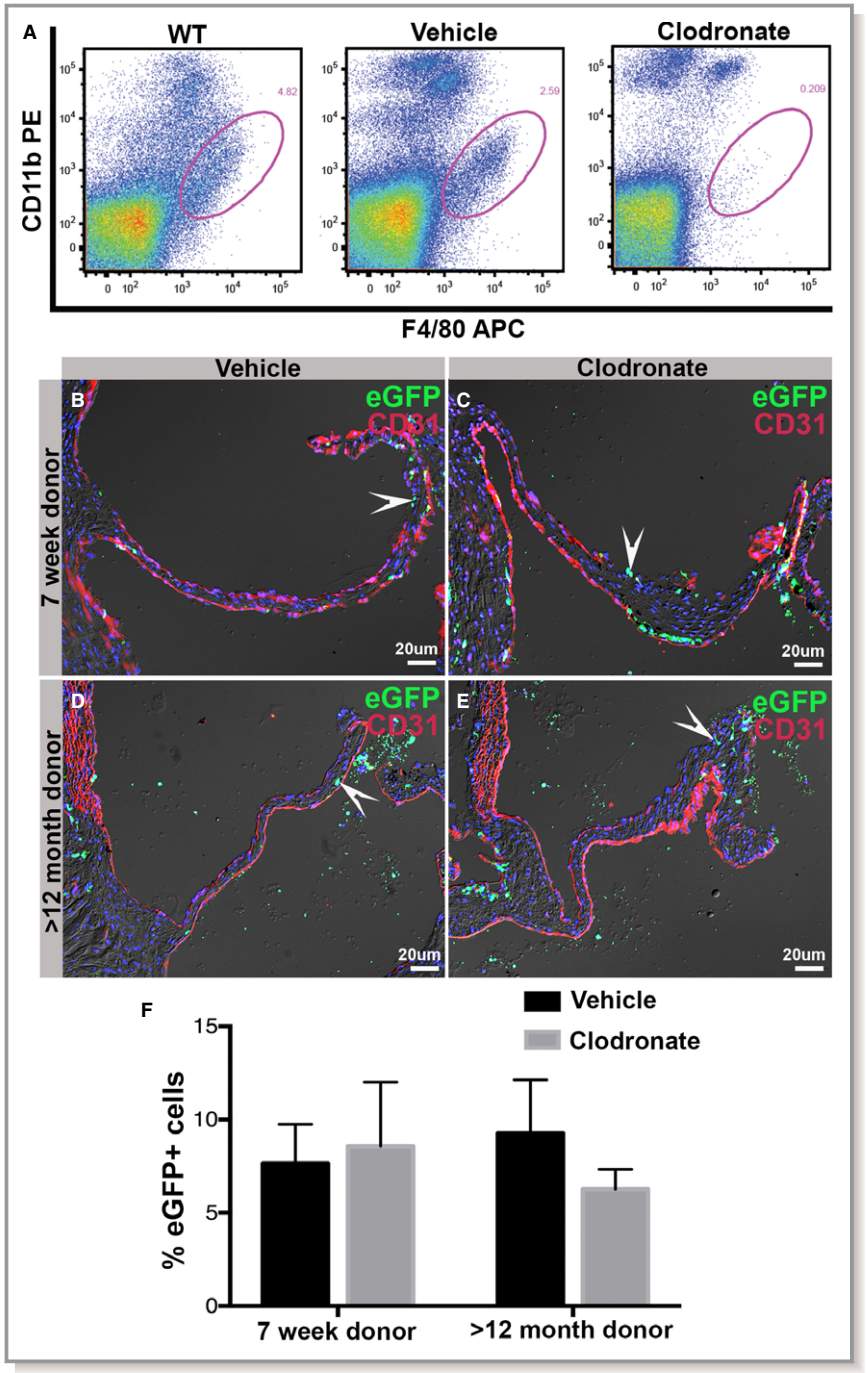

Figure 5. Transplanted bone-marrow–derived cells within the aortic valve do not differentiate into mature macrophages. A, Representative flow cytometry plots of isolated splenocytes from wild-type, vehicle (empty liposome), and liposomal Clodronate-treated 7-week-old recipient BMT mice, stained for CD11b-PE and F4/80-APC. The gated (circled) area shows the depleted macrophage population in Clodronate-treated mice. B through E, Immunofluorescent staining for eGFP (green), to show transplanted cells, and CD31 (red), to mark the VEC compartment, of vehicle (B and D) or Clodronate-treated (C and E) 7-week-old recipients who received bone marrow from 7-week-old donors (B and C) or >12-month-old donors (D and E). F, Percentage of $eGFP^+$ cells in the aortic valves of vehicle and Clodronate-treated BMT recipients. n=at least 3. BMT indicates bone marrow transplant; GFP, enhanced green fluorescent protein; VEC, valve endothelial cell; WT, wild type.

quiescent, but then differentiates accordingly to maintain or replenish the valve cell populations as necessary in response to injury, disease, or severe wear and tear. Recent work by Bischoff et al utilized a myocardial infarction ovine model to induce mitral valve fibrosis and observed increased expression of CD45 within the valve endothelium. In this model of valve disease, the group suggested that CD45-positive cells are important in the mitral valve adaptive response following myocardial infarction, potentially by undergoing endothelial-to-mesenchymal transformation that may be beneficial early, but contributing to pathogenesis later.[16]

Here, we have provided evidence that CD45-derived cells to homeostatic valve leaflets is a normal process, and cells of the hematopoietic lineage maintain CD45 expression and rarely express valve cell markers exclusively. In addition, we have demonstrated that the efficiency of bone marrow cell engraftment to the aortic valve following transplantation is age-dependent. However, it is recognized that, despite no overt effects of irradiation, the contribution of cells may be affected by this procedure. Nonetheless, this study does support previous work stemming back to 1998 showing contribution of these cells to the valves; however, we have yet to determine the function and requirement of these cells under healthy and diseased conditions. Providing such insights will significantly advance the field and profoundly impact the future of therapeutic applications.

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Disclosures

None.

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