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Authors

Perez-Cunningham, Jessica
Boyer, Scott W
Landon, Mark
et al.

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Hematopoietic stem cell-specific GFP-expressing transgenic mice generated by genetic excision of a pan-hematopoietic reporter gene

Jessica Perez-Cunningham, Scott W. Boyer, Mark Landon, and E. Camilla Forsberg

Department of Biomolecular Engineering, Institute for the Biology of Stem Cells, University of California, Santa Cruz, CA

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Selective labeling of specific cell types by expression of green fluorescent protein (GFP) within the hematopoietic system would have great utility in identifying, localizing, and tracking different cell populations in flow cytometry, microscopy, lineage tracing, and transplantation assays. In this report, we describe the generation and characterization of a new transgenic mouse line with specific GFP labeling of all nucleated hematopoietic cells and platelets. This new "Vav-GFP" mouse line labels the vast majority of hematopoietic cells with GFP during both embryonic development and adulthood, with particularly high expression in hematopoietic stem and progenitor cells (HSPCs). With the exception of transient labeling of fetal endothelial cells, GFP expression is highly selective for hematopoietic cells and persists in donor-derived progeny after transplantation of HSPCs. Finally, we also demonstrate that the loxP-flanked reporter allows for specific GFP labeling of different hematopoietic cell subsets when crossed to various Cre reporter lines. By crossing Vav-GFP mice to Flk2-Cre mice, we obtained robust and highly selective GFP expression in hematopoietic stem cells (HSCs). These data describe a new mouse model capable of directing GFP labeling exclusively of hematopoietic cells or exclusively of HSCs.

There have been many efforts to generate transgenic mice with transgene expression exclusively in the hematopoietic compartment [1]. The *vav1* gene has been the focus of many such studies, because it is highly expressed throughout hematopoietic development from embryonic day 11.5 (e11.5) through adulthood [2]. There appears to be very limited expression in other tissues in the adult mouse, with the exception of the developing tooth bud [2]. Vav1 has been shown to activate the Rac/Jun kinase pathway, and gene disruption assays have shown it to be essential for signaling through the antigen receptors of lymphocytes [3–5]. Interestingly, even though Vav1 is highly expressed throughout the hematopoietic system, it is not essential for the development of blood cells in general [6].

Offprint requests to: E. Camilla Forsberg, PhD, Department of Biomolecular Engineering, Institute for the Biology of Stem Cells, University of California, MS: SOE 2, 1156 High Street, Santa Cruz, CA 95064; E-mail: cforsber@soe.ucsc.edu

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The unique expression pattern of the *vav1* gene has led to the generation of several *vav*-driven cre mouse lines and *vav*-driven direct reporter mouse lines [7–12]. These mouse lines have generally had great success in labeling the hematopoietic compartment with minimal off-target expression. Of particular note is Stadtfeld and Graf's model in which Cre recombinase is driven with promoter and enhancer elements of the *vav* gene [10]. When crossed to a stop-lox-yellow fluorescent protein (YFP) reporter line, this model accomplished almost 100% labeling in all nucleated bone marrow (BM) cells and platelets in adult mice. They also found that nearly all $cKit^+Lin^-Sca^+$ (KLS) cells were labeled in the e13.5 fetal liver, and approximately one half of $CD45^+$ (hematopoietic) cells from the e10.5 fetal liver were reporter positive [10]. Although this mouse line demonstrated great success in labeling the entire hematopoietic compartment, it does not allow for the resolution of specific cell populations within the hematopoietic lineage needed for experiments such as lineage tracing from hematopoietic stem cells (HSCs) and/or hematopoietic stem progenitor cells (HSPCs) or localization of HSCs/HSPCs. To enable fluorescent labeling of specific hematopoietic cell populations, we modified Stadtfeld's construct

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so that the *vav* enhancer/promoter elements drive a fluorescent reporter that can be excised in specific hematopoietic cell subsets using Cre-mediated recombination. This new mouse line, called "Vav-GFP," allows for two levels of specificity: first, the fluorescent reporter is under control of *vav* promoter elements, and second, it can be crossed to a multitude of Cre lines to drive excision of the reporter and thereby restrict fluorescence to a desired population of HSCs or HSPCs.

In this study, we characterized the fluorescence of the Vav-GFP mouse line in BM and peripheral blood (PB) in both adult and fetal mice. In addition, we showed that the Vav-GFP cells can be distinguished from wild-type (WT) host cells after transplantation, because this is a likely application of the new mouse line. Finally, we also crossed the Vav-GFP mice to a Flk2-driven Cre mouse line to achieve targeted labeling exclusively of HSCs within the BM compartment [13,14]. These data show that the Vav-GFP mouse line generated here represents a novel tool with which to interrogate HSC differentiation and trafficking by providing hematopoietic-specific expression of a reporter construct under control of Cre-mediated recombination.

Methods

Generation of Vav-GFP transgenic mice

Enhanced green fluorescent protein (EGFP) and HcRed were cloned in the pZ/EG plasmid [15], replacing lacZ and EGFP, respectively, to generate an EGFP-stop-loxP-HcRed-loxP reporter construct. The reporter fragment was then migrated into the vavINS-Cre-IRES-YFP plasmid [10] after SfiI and Not I digestion and three-piece ligation to replace Cre-IRES-YFP with the EGFP-stop-loxP-HcRed-loxP reporter fragment. The vector was linearized and injected into pronuclei of C57BL/6 mice at the University of California–Santa Cruz (UCSC) transgenic facility. Multiple founders were used to establish a colony, but founder lines were not analyzed separately. Characterization of this line revealed no HcRed fluorescence after floxing. All mice were maintained and investigated in the UCSC vivarium according to institutional animal care and use committee-approved protocols.

Flow cytometry analysis

BM and PB cells were isolated, processed, and analyzed using a four-laser FACSAria cell sorter or an LSRII flow cytometer (BD Biosciences, San Jose, CA) as described previously [16–18]. FlowJo Software (Ashland, OR) was used for data analysis and display. Mean fluorescence intensity was determined for each cell population by calculating the average intensity for the entire population of both experimental and WT controls and then subtracting the average WT intensity from each experimental replicate. Cell populations were defined by the following cell surface phenotypes: HSCs ($Lin^-Sca1^+c\text{-}kit^+CD48^-Slamf1^+Flk2^-$), multipotent progenitors (MPPs; Lin^-Sca1^+ $c\text{-}kit^+CD48^+Slamf1^-Flk2^+$), common myeloid progenitors ($Lin^-Flk2^-Sca1^-$ $c\text{-}kit^+FcgR^{mid}CD34^{mid}$), granulocyte/macrophage (GM) progenitors ($Lin^-Flk2^-Sca1^-$ $c\text{-}kit^+FcgR^{hi}CD34^{hi}$), megakaryocyte/erythroid progenitors ($Lin^-Flk2^-Sca1^-$ $c\text{-}kit^+FcgR^{lo}CD34^{lo}$), CLP ($Lin^-Sca1^{mid}$ $c\text{-}kit^{mid}Flk2^+IL7Ra^+$), GMs ($Ter119^-Mac1^+Gr1^+CD3^-B220^-$), platelets ($FSC^{lo}Ter119^-CD61^+$), B-cell progenitors ($Ter119^-Mac1^-Gr1^-CD3^-B220^+$), T-cell progenitors ($Ter119^-Mac1^-Gr1^-CD3^+B220^-$), and erythroid progenitors ($B220^-$, $CD3^-$,$Mac1^-$,$Gr1^-$, $Ter119^+$,$Cd71^+$). The lineage mixture consisted of antibodies recognizing CD3, CD4, CD5, CD8, B220, Gr1, Mac1, and Ter119. Mac1 antibodies were excluded from the lineage mixture when analyzing fetal progenitors [19].

Transplantation assays

CD117-enriched BM cells were isolated and double sorted from Vav-GFP mice using a FACSAria III cell sorter and then transplanted into sublethally irradiated (500 rads) WT mice (C57BL/6) [13]. One thousand KLS cells were transplanted into each recipient, and PB or BM was analyzed for 24 weeks after transplantation.

Nonhematopoietic cell isolation

Whole organs were dissected from Vav-GFP and WT C57BL/6 mice and homogenized using a mortar and pestle for all organs. Cell suspensions were passed through 70-micron filters and stained with anti-CD45 and Ter119 antibodies to exclude hematopoietic cells from analysis. Endothelial cells (ECs) were isolated as described previously [17]. Briefly, BM cells were isolated from Vav-GFP and WT mice and digested in a 3 mg/mL collagenase solution for 1 hour. Similarly, e14.5 fetal livers were isolated from Vav-GFP and WT embryos and digested with a 1 mg/mL collagenase solution. Samples were then filtered and stained with anti-CD45, CD31, Sca1, Tie-2, and Vcam1 antibodies and analyzed using flow cytometry.

Results and discussion

Characterization of reporter expression in hematopoietic cell populations

Our goal was to generate a dual-purpose transgenic mouse line that allows for pan- hematopoietic or in combination with select Cre-expressing mouse lines, labeling of a subset of HSCs/HSPCs. To generate Vav-GFP mice, we used the murine regulatory elements of the *vav* gene to drive expression of a dual color reporter. A vector consisting of Vav regulatory elements and loxP-flanked EGFP was linearized and injected into pronuclei of C57BL/6 mice (Fig. 1A). In this model, GFP is expressed until Cre-mediated recombination causes excision of GFP and a stop codon (Figs. 1A and 5A).

To investigate the ability of the reporter construct to label hematopoietic cells fluorescently, HSPCs and mature cell populations were isolated from BM and PB of Vav-GFP mice. Flow cytometry analysis revealed reporter expression in all HSPCs and mature cells, including platelets and erythroid progenitors, but not in mature circulating red blood cells (RBCs; Fig. 1B and 1C). All HSCs, MPPs, and myeloid progenitors displayed strong GFP expression that was clearly distinguishable from WT control cells (Fig. 1B and 1D). Mature BM cell populations also expressed GFP, although at lower fluorescent intensities

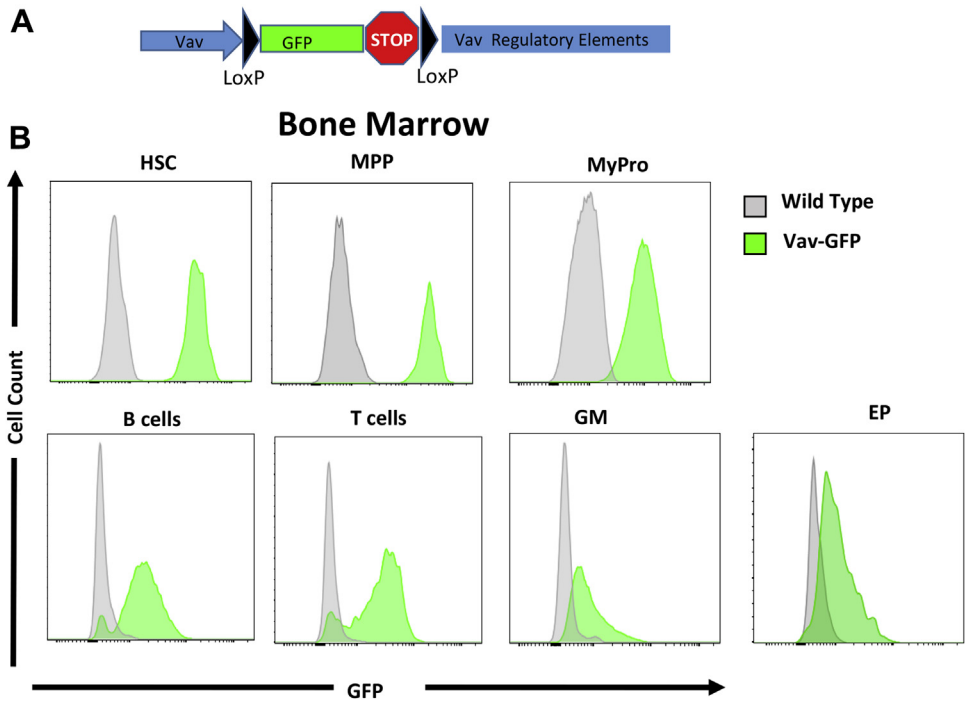

Figure 1. Vav-driven GFP expression labels all nucleated hematopoietic cell types and platelets in BM and PB. (**A**) Schematic diagram of the Vav-GFP reporter construct. (**B–G**) Reporter expression in BM and PB cells in Vav-GFP mice was tested by flow cytometry. (**B**) Representative flow cytometer histograms indicating GFP expression levels in the indicated BM cell populations of Vav-GFP (*green*) and WT (*gray*) mice. (Color version of figure available online.)

compared with HSPCs (Fig. 1B, 1D, and 1E). In the PB, virtually all hematopoietic cells expressed GFP at levels readily distinguishable from WT control cells (Fig. 1C, 1F, and 1G). Notably, GFP expression was robust in platelets, but was not detected in circulating RBCs. In addition, reporter expression in circulating GM was significantly higher than in BM-resident GM (Supplementary Figure E1, online only, available at www.exphem.org), which may be due to differences in Vav expression between these populations. Together, these data show strong reporter expression in all BM and circulating hematopoietic cells, with the exception of RBCs, making the Vav-GFP transgenic mouse a valuable tool for studying the blood system.

Reporter expression in adult mice is restricted to hematopoietic cells

The Vav-GFP model was designed only to fluoresce in hematopoietic cells and in no other tissues or cell types. To test the reporter specificity, we investigated GFP expression in nonhematopoietic cells of the brain, liver, heart, and lungs. Whole organs were isolated, prepared into single-cell suspensions, and stained with pan-hematopoietic (CD45) and erythroid (Ter119) markers to exclude hematopoietic cells. Each organ was then analyzed by flow cytometry, and $CD45^{-}Ter119^{-}$ cells were tested for GFP expression. Although GFP expression was detected readily in co-isolated $CD45^{+}$ cells, there was no detectable off-target GFP expression found in nonhematopoietic cells in any of the organs surveyed (Fig. 2A and 2B).

The most likely population of cells to exhibit off-target expression in the Vav-GFP model are ECs, because other studies using *vav* regulatory elements have reported mixed results of off-target expression in ECs. For example, Georgiades et al. showed that all $CD31^{+}$ cells were labeled with b-galactosidase with their vav-cre line, whereas Ogilvy et al. reported no vav-driven hCD4 in nonhematopoietic tissues by immunohistochemical analysis [7,8]. To test endothelial GFP expression in our model, we isolated $CD45^{-}Ter119^{-}CD31^{+}Sca1^{+}$ ECs from the BM of Vav-GFP mice. In all ($n = 6$) but one mouse surveyed, GFP expression was undetectable in BM ECs (Fig. 2C and 2D). One mouse had approximately 5% of its ECs labeled with GFP, but no other mouse surveyed, including littermates, showed similar expression patterns.

Vav-GFP is expressed selectively in fetal hematopoietic cells

Given that *vav* expression has been detected in the embryo as early as e11.5 [2], we wanted to test GFP expression in embryonic hematopoietic cells in Vav-GFP mice. Most but not all (~85%) of $CD45^{+}ckit^{+}$ hematopoietic cells isolated from the caudal half of e11.5 Vav-GFP embryos displayed robust GFP expression (Fig. 3A). The partial labeling may be due to inadequate accumulation of GFP because the transgene is just beginning to be expressed at this time point, or it could be due to heterogeneity of the cell types included in the $CD45^{+}ckit^{+}$ phenotypic compartment.

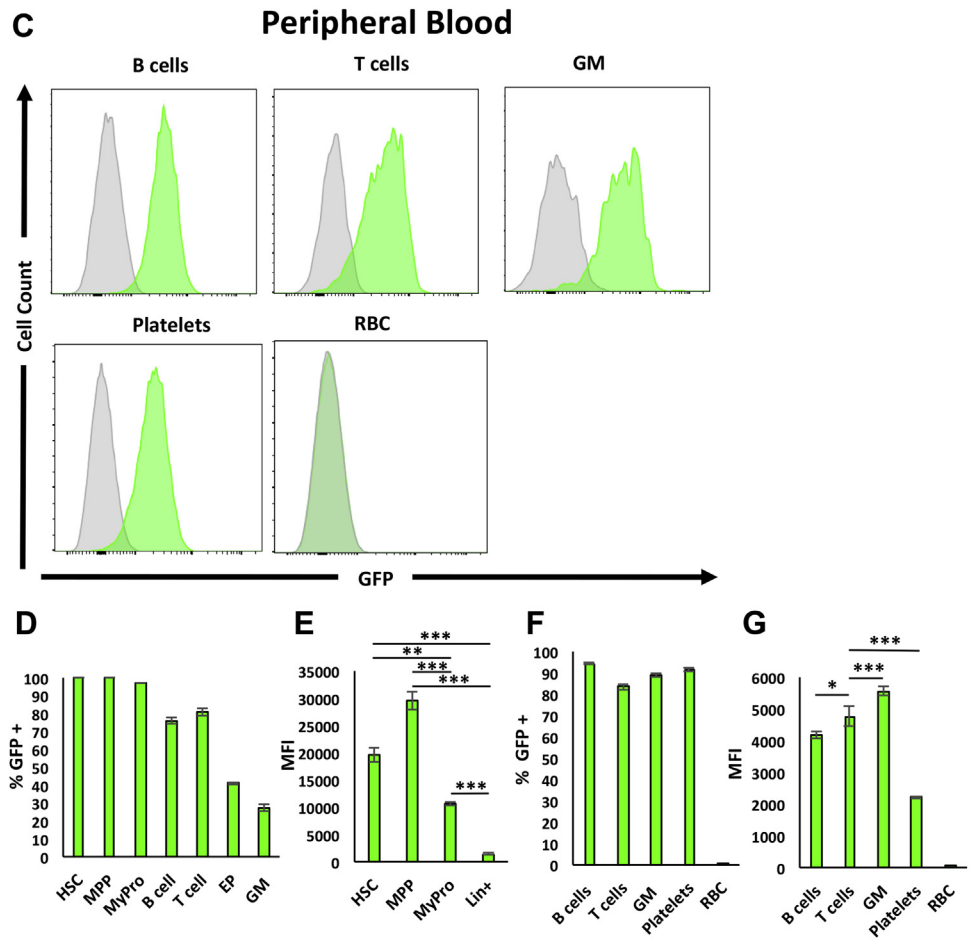

Figure 1. (*continued*) (**C**). Representative flow cytometry histograms indicating GFP expression levels in PB cells in Vav-GFP and WT mice. (**D**) Proportion of reporter expressing cells in hematopoietic subsets isolated from the BM of Vav-GFP mice is shown as the percentage of cells positive for GFP. (**E**) MFI indicates intensity of GFP reporter expression in the indicated BM cell populations. (**F**) Proportion of reporter expressing cells in hematopoietic subsets isolated from the PB of Vav-GFP mice shown as the percentage of cells positive for GFP. (**G**) MFI indicates intensity of reporter expression in the indicated PB cell populations. In all histograms, *gray* bars represent MFI in WT samples and *green* bars represent MFI in Vav-GFP mice. $n = 3–7$ mice per experiment. Statistically significant differences were determined by an equal variance t test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. EP = erythroid progenitor; MFI = mean fluorescence intensity; MyPro = myeloid progenitor.

Because both progenitor and mature cell populations have been well characterized in fetal livers at e14.5, we investigated the GFP expression of hematopoietic cell subsets at this stage. We observed strong labeling in all hematopoietic cells surveyed, including HSPCs and mature cells (Fig. 3B). Compared with their adult counterparts, the level of GFP expression varied in some cases, with fetal HSCs displaying lower GFP intensity than adult HSCs, whereas embryonic B cells appeared brighter than their adult equivalents (Fig. 3B).

To test the specificity of GFP expression in fetal Vav-GFP mice, we isolated ECs from e14.5 fetal livers. In contrast to the lack of GFP expression in adult ECs, a large proportion (~50%) of ECs in the e14.5 fetal liver exhibited GFP expression (Fig. 3C). Their EC identity was confirmed by costaining with anti-Tie2 and Vcam1 antibodies. GFP expression in ECs is clearly transient, because it was not detected in the adult and may reflect a brief period of vav expression by early endothelial cells or progenitors. This off-target expression is unlikely to affect adult studies with our Vav-GFP mouse line, but it may require exclusion of ECs to study developmental hematopoiesis in these mice. For example, the ECs can be excluded from flow cytometry assays by either MECA 32 expression or lack of CD45 expression. Conversely, endothelial GFP expression may be used to investigate fetal EC populations or the relationship between endothelial and hematopoietic development.

Robust reporter expression is detected after transplantation

To test whether GFP expression remains robust during reconstitution after transplantation, we performed transplantations with KLS cells isolated from Vav-GFP mice. All mature cell and progenitor lineages surveyed in recipient mice showed clear separation by GFP expression

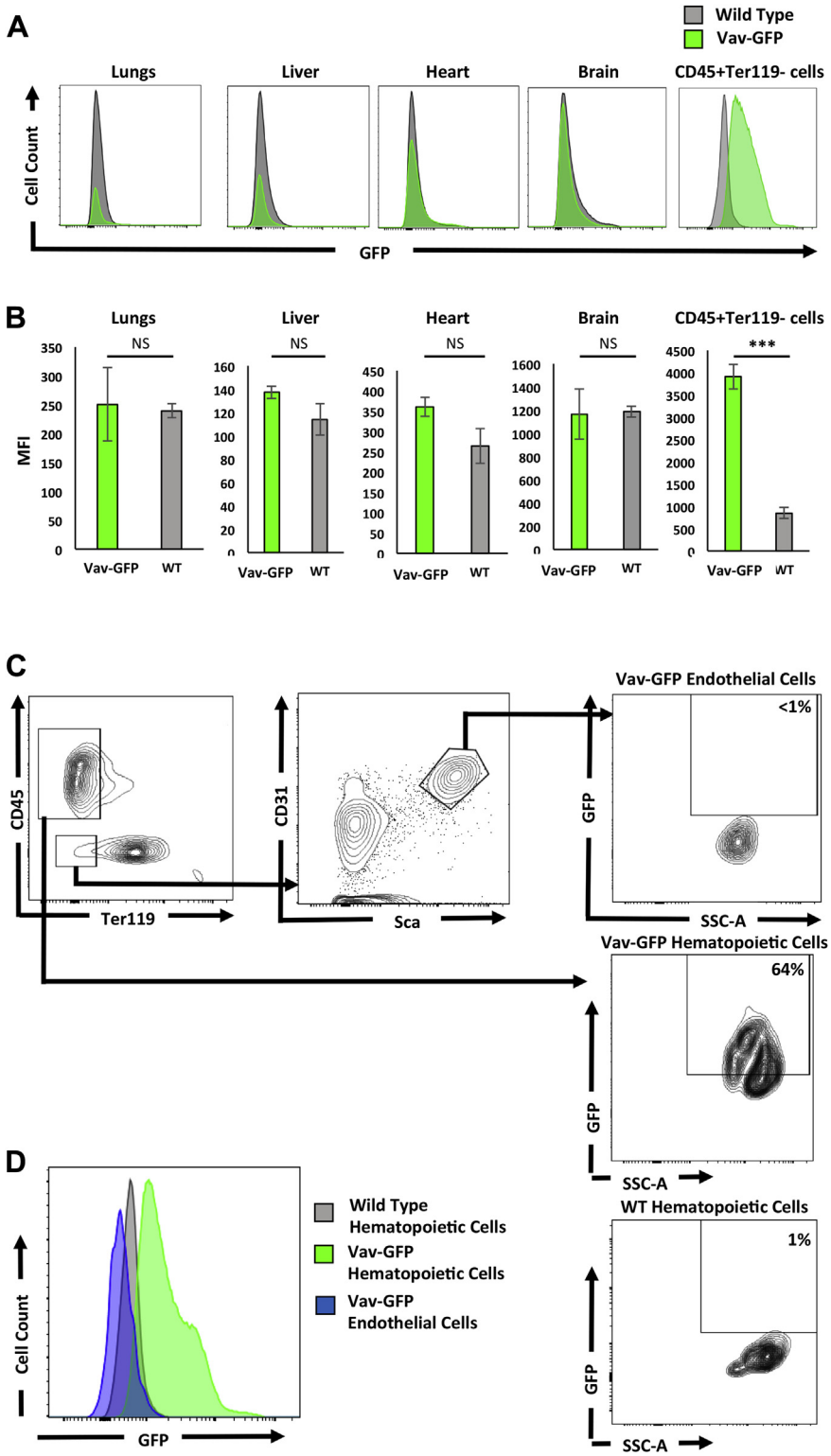

Figure 2. GFP expression is largely restricted to hematopoietic cells. (**A, B**) Vav-GFP reporter expression was observed in hematopoietic ($CD45^+$), but not nonhematopoietic ($CD45^-$), cells in the lungs, liver, heart, or brain. Whole organs were isolated from adult Vav-GFP mice and processed into single-cell suspensions before antibody staining and analysis by flow cytometry. Representative histograms (**A**) and MFI (**B**) of GFP expression reveal no statistical differences between WT and Vav-GFP mice for nonhematopoietic cells. $n = 3$ mice. (**C, D**) Endothelial cells ($CD45^-$, $Ter119^-$, $Sca1^+$, $CD31^+$) isolated from the BM of Vav-GFP mice did not express GFP, whereas concurrently isolated $CD45^+$ hematopoietic cells demonstrated robust labeling. (**C**) Flow cytometric gating strategy used to define endothelial and hematopoietic cells. (**D**) Histogram plot showing representative GFP expression profiles from hematopoietic and endothelial cells isolated from WT and Vav-GFP mice. Percentages indicate representative frequencies of GFP^+ cells for each cell population. $^{***}p < 0.001$. $n = 6$ mice from two independent experiments. NS = not significant. (Color version of figure available online.)

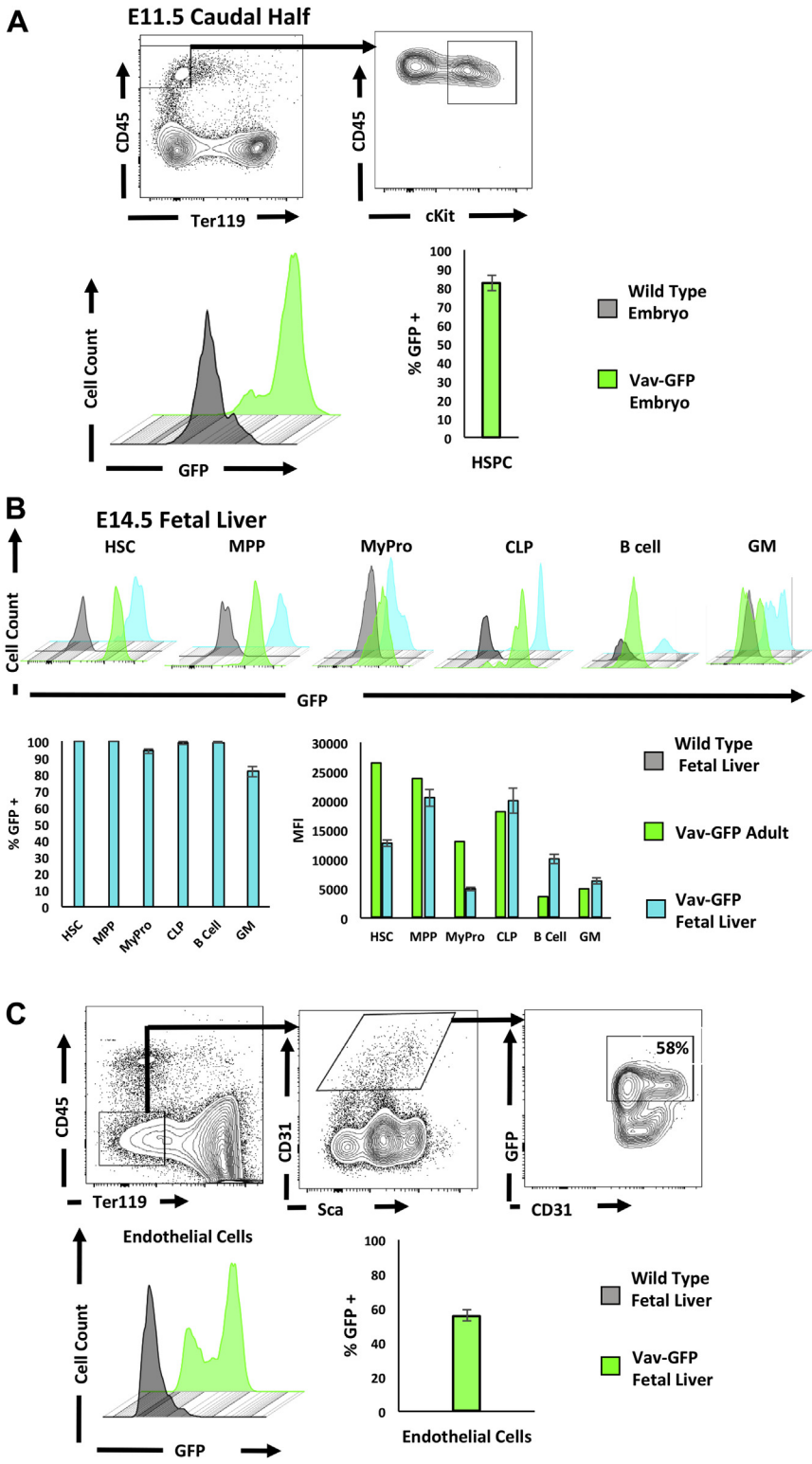

Figure 3. Vav-GFP reporter expression marks hematopoietic cells across development (**A**) Vav-GFP reporter expression was observed as early as e11.5. Representative gating strategy, histograms, and quantification by flow cytometric analysis of reporter activity in HSPCs ($CD45^+$, $ckit^+$, $Ter119^-$) in the caudal half of e11.5 Vav-GFP and WT control embryos. $n = 7$ embryos representing two independent experiments. (**B**) Vav-GFP reporter expression was detected in stem, progenitor, and mature cells isolated from e14.5 fetal livers of Vav-GFP embryos. Representative histograms depict flow cytometric analysis of progenitor and mature cell populations. $n = 3$ embryos. *Gray* histograms represent WT embryos; *green* histograms represent Vav-GFP adult cells, and *blue* histograms represent Vav-GFP fetal liver cells. (**C**) Reporter expression was detected in a proportion of endothelial cells during development. Gating strategy, representative histogram, and proportion of GFP^+ endothelial cells ($CD45^-$, $Ter119^-$, $CD31^+$, $Sca1^{mid}$) isolated from e14.5 fetal liver of Vav-GFP and WT embryos are shown. $n = 13$ embryos representing two independent experiments. (Color version of figure available online.)

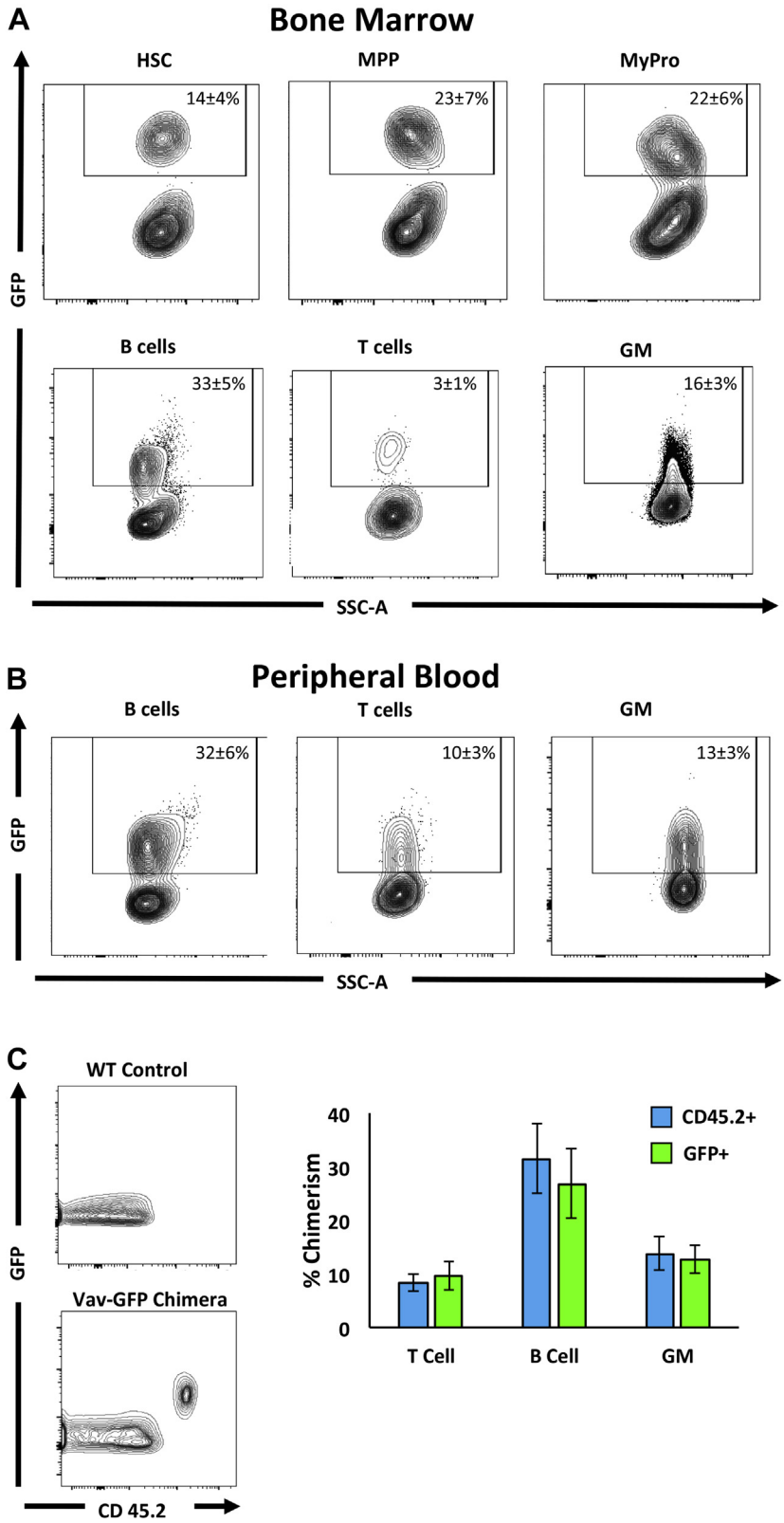

Figure 4. Robust Vav-GFP expression is maintained in donor-derived cells after transplantation. Adult KLS cells were isolated from Vav-GFP mice and transplanted into sublethally irradiated WT recipients. PB and BM were analyzed 24 weeks after transplantation to assess donor contribution and expression of the Vav-GFP reporter. (**A**) Representative flow plots depicting reporter expression in all mature and progenitor populations analyzed at 24 weeks. (**B**) Representative flow plots also show reporter expression in mature PB subsets at 24 weeks. (**C**) Flow plots showing live Ter119$^-$ cells from an untransplanted recipient (CD45.1) and chimeric Vav-GFP (CD45.2). All observed donor cells were double-positive for CD45.2 and GFP. This is quantified on the right for mature PB cells with CD45.2$^+$ cells shown in blue and GFP$^+$ cells shown in green. Data represent four biological replicates. (Color version of figure available online.)

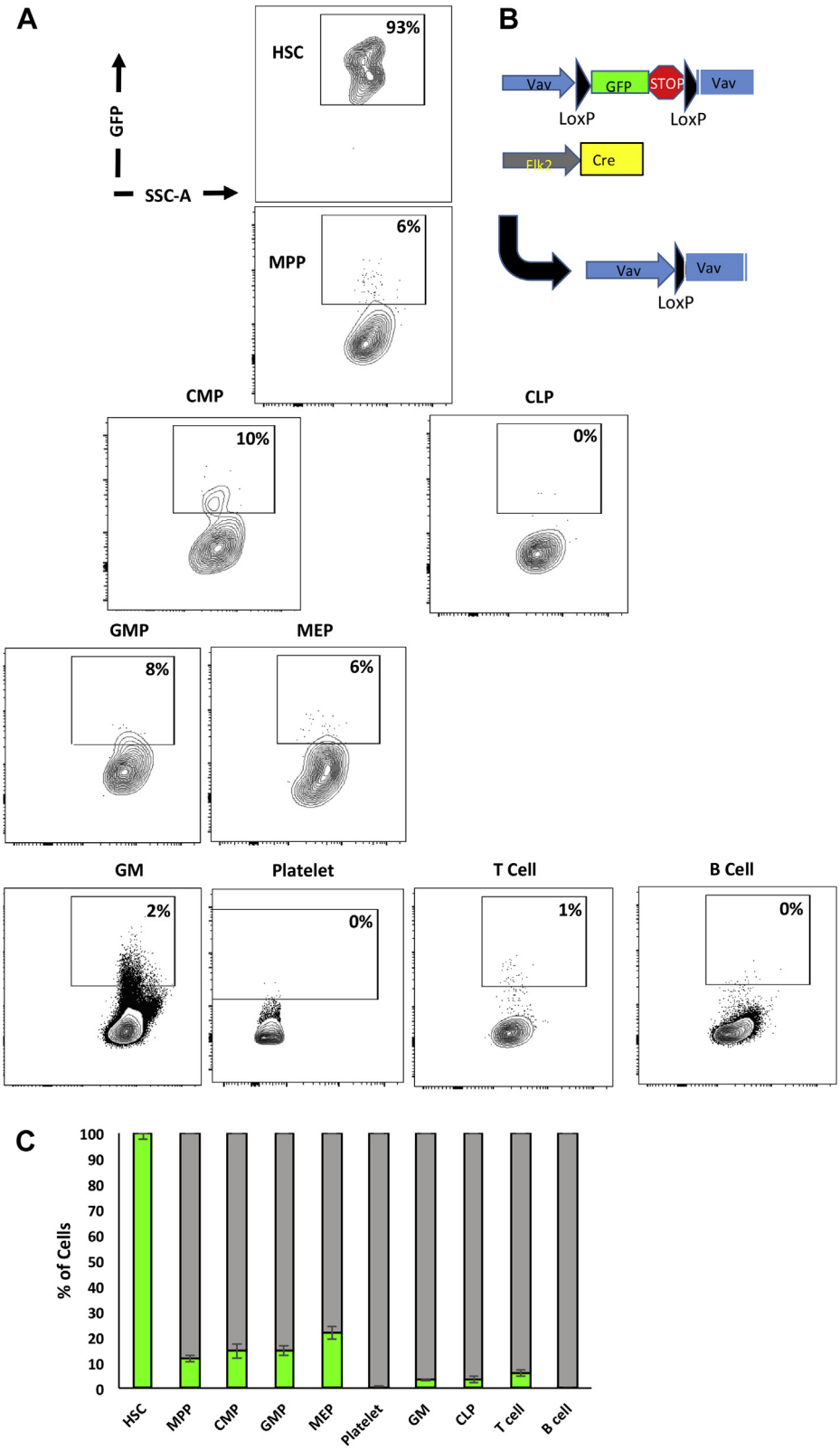

Figure 5. Flk2-Cre-mediated excision of Vav-driven GFP labels HSCs selectively. Vav-GFP mice were crossed to Flk2-Cre mice to assess the ability to label HSCs specifically. (**A**) Schematic showing excision of GFP reporter by Cre-mediated recombination. (**B**) Representative flow cytometric analysis of reporter expression in hematopoietic subsets isolated from the BM of Vav-GFP/Flk2-Cre double-transgenic mice. Numbers indicate the frequency of GFP-expressing cells in each gated cell population of a representative Vav-GFP/Flk2-Cre mouse. (**C**) Percentage of cells expressing GFP in each stem, progenitor, and mature population in the BM. Data represent 11 mice from three independent experiments.

between host and donor populations (Fig. 4A and 4B). Analysis of live, Ter119$^-$ cells in chimeric animals showed that all donor-derived cells (CD45.2$^+$) were also GFP$^+$. This result verifies that the transgene clearly labels donor cells upon transplantation and supports the utility of the Vav-GFP mice to track transplanted hematopoietic cells in reconstitution assays.

HSC-specific GFP expression accomplished by crossing Vav-GFP and Flk2-Cre lines

The flanking of GFP with loxP sites enables excision of the reporter gene in desired cell populations. To test whether HSC-specific labeling could be accomplished, we crossed the Vav-GFP mice to Flk2-Cre transgenic mice [20]. We and others have shown previously that Flk2 is expressed by MPPs [16,21–23] and that Flk2-Cre labeling combined with a color-switch model is capable of labeling the entire hematopoietic system downstream of the HSC [13,14]. By crossing Vav-GFP mice to the Flk2-Cre line, we anticipated that all HSCs would be labeled with GFP and that all downstream progenitors and mature cells would lack GFP expression due to excision of the reporter gene in Flk2$^+$ progenitor cells [24]. In addition, nonhematopoietic cells would lack GFP expression due to the hematopoietic-specific expression of Vav-driven transgenes (Figs. 1 and 2). Consistent with our previous findings, we observed strong and highly selective GFP expression in HSCs, whereas the vast majority of hematopoietic progenitors and mature cells were unlabeled (Fig. 5). Expression of GFP in a small proportion of downstream populations is a result of incomplete floxing from the specific Cre mouse used. In the case of Flk2-Cre mice, the recombination efficiency varies between individual Flk2-Cre mice [13,14]. Therefore, the combination of Vav-GFP and Flk2-Cre enables very bright, highly specific GFP labeling of HSCs.

In conclusion, the Vav-GFP mouse line directs GFP expression exclusively in the hematopoietic system, with the option of differentiation stage specificity by Cre-mediated recombination. We detected strong GFP labeling of all hematopoietic cell types assayed, except for RBCs, with minimal off-target expression in adult tissues. We also detected strong labeling of hematopoietic cells in the developing embryo, with limited off-target GFP expression in ECs. Transplantation experiments demonstrated GFP expression can be used to distinguish donor-derived cells from host cells in hematopoietic reconstitution assays. We showed that the loxP elements are functional for Cre-mediated recombination by crossing the Vav-Cre mice with an Flk2-Cre line, which led to highly selective GFP labeling of HSCs in the BM compartment. The Vav-GFP × Flk2-Cre cross provides an excellent example of how the Vav-GFP line can be used for lineage-tracing studies and offers direct visualization of HSC in adult BM for *in situ* assays and for tracking HSC migration upon transplantation or mobilization. Our mice can also be crossed to commercially available floxed-stop-reporter mice to achieve labeling of HSC progeny with a second color. For example, a cross to a Rosa26-lox-stop-tomato mouse would result in mice with GFP$^+$ HSCs, Tomato$^+$ hematopoietic progenitor and mature cells, and unlabeled nonhematopoietic cells. Collectively, our data show that the Vav-GFP mouse line, alone or by breeding to specific Cre lines to obtain selective GFP labeling in hematopoietic subpopulations, represents a novel tool for interrogating the hematopoietic system without fluorescence interference from nonhematopoietic cells. Our strategy to achieve HSC-specific reporter expression can be used for the expression of any other transgene specifically in HSCs to assess transgene function exclusively in HSCs.

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Conflict of interest disclosure

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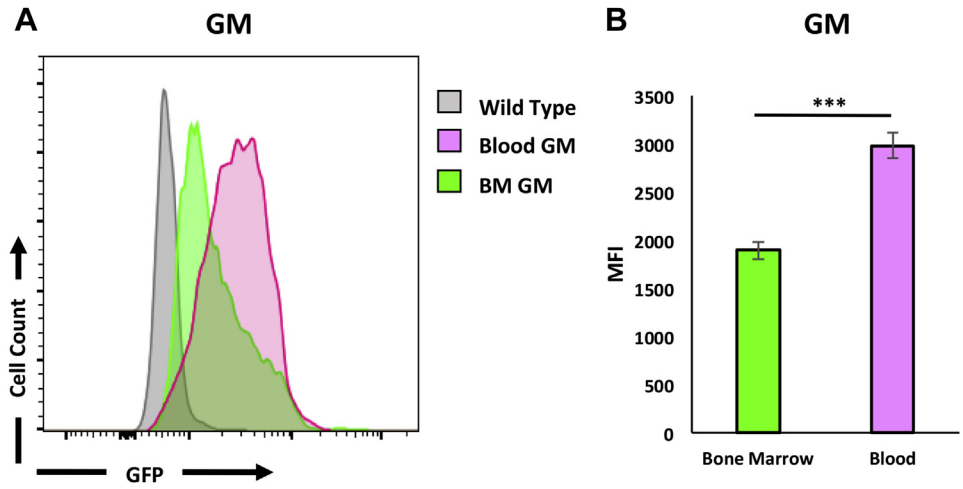

Supplementary Figure E1. Circulating GM cells show significantly higher reporter expression than BM-resident GM cells. (**A**) Flow cytometry histograms showing GFP expression levels of WT-circulating GM cells (*gray*), BM-resident Vav-GFP GM cells (*green*), and circulating Vav-GFP GM cells (*magenta*). (**B**) Mean fluorescent intensity of BM-resident and circulating GM cells in Vav-GFP mice. ***$p < 0.001$.