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Dual roles for bone marrow–derived Sca-1 cells in cardiac function

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ABSTRACT: Recruitment of stem cells from the bone marrow (BM) is an important aspect of cardiac healing that becomes inefficient with age. We investigated the role of young stem cell antigen 1 (Sca-1)-positive BM cells on the aged heart by microarray analysis after BM reconstitution. Sca-1$^+$ and Sca-1$^-$ BM cells from young green fluorescent protein (GFP)-positive mice were used to reconstitute the BM of aged mice. Myocardial infarction (MI) was induced 3 mo later. GFP$^+$ cells were more abundant in the BM, blood, and heart of Sca-1$^+$ mice, which corresponded to preserved cardiac function after MI. At baseline, Sca-1$^+$ BM reconstitution increased cardiac expression of serum response factor, vascular endothelial growth factor A, and myogenic genes, but reduced the expression of *Il-1β*. After MI, inflammation was identified as a key difference between Sca-1$^-$ and Sca-1$^+$ groups, as cytokine expression and cell surface markers associated with inflammatory cells were up-regulated with Sca-1$^+$ reconstitution. Mac-3 and F4/80 staining showed that the postinfarction heart was composed of a mixture of GFP$^+$ (donor) macrophages, GFP$^-$ (host) macrophages, and GFP$^+$ cells that did not contribute to the macrophage population. This study demonstrates that Sca-1$^+$ BM cells regulate cardiac healing though an acute inflammatory response and also before injury by stimulating formation of a beneficial cardiac niche.—Tobin, S. W., Li, S.-H., Li, J., Wu, J., Yeganeh, A., Yu, P., Weisel, R. D., Li, R.-K. Dual roles for bone marrow–derived Sca-1 cells in cardiac function. FASEB J. 31, 2905–2915 (2017). www.fasebj.org

KEY WORDS: aging · cardiovascular disease · stem cells

Cardiovascular disease remains an important clinical problem in the Western world. After cardiac injury such as myocardial infarction (MI), cardiomyocytes undergo significant cell death. In response to the ischemic insult, different stem cell populations, particularly from the bone marrow (BM), have been reported to home to the site of injury and proliferate to improve cardiac function and survival (1). Activation and recruitment of these stem cells to the heart is reduced with age, and repair of heart function is therefore limited in the elderly (2). The importance of endogenous stem cell mobilization in patients with cardiovascular disease was recently highlighted by Al Mheid *et al.* (3), who demonstrated that chronic exposure to risk factors together with age reduces the number of circulating progenitor cells. Thus, recruitment and activation of progenitor cells contributes to preservation of heart function after injury, but this process becomes incomplete with aging. Two important features of circulating progenitor cells are their involvement in inflammation and angiogenesis (4), which occur sequentially after heart injury and become impaired with aging (5).

Emerging evidence indicates that a youthful environment enhances endogenous regeneration and that systemic factors profoundly influence tissue aging (6). Although this leads to the attractive potential that age-associated deleterious change can be reversed, there are different consequences associated with aging, such as proliferation and inflammation that may require independent molecular approaches. For example, Jaskelioff *et al.* (7) suggested that aging is reversible as restoration of telomere integrity reversed the age-related decline in the central nervous system and other organs. A study by Zhang *et al.* (8) raised the possibility that controlling inflammation in age-related disorders, including heart disease, could have important

ABBREVIATIONS: BM, bone marrow; DAVID, Database for Annotation, Visualization, and Integrated Discovery; FDR, false discovery rate; FGF10, fibroblast growth factor 10; GFP, green fluorescent protein; GO, Gene Ontology; *Jmjd6*, Jumonji domain-containing 6; LAD, left coronary artery; MI, myocardial infarction; PE, phycoerythrin; qPCR, quantitative PCR; Sca-1, stem cell antigen 1; *Setd8*, SET domain-containing 8; *Smarca2*, SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin, subfamily a, member 2; SRF, serum response factor; *Tnfrsf12a*, TNF receptor superfamily, member 12a; *Vegfa*, vascular endothelial growth factor A

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benefits in delaying the aging of various organs. It was also recently shown that simple blood exchange between young and old rodents can rapidly modulate tissue health; however, the negative impact of old blood in the younger animal was greater than the positive outcome of young blood transfer to old mice (9).

A number of circulating cells arise from the BM that may contribute to the endothelial, inflammatory, and cardiac progenitor cell populations upon reaching the heart (10, 11). Interestingly, young to old BM reconstitution can restore progenitor cells in the BM and the myocardium in aged mice and can preserve heart function after MI (2). Our group has recently shown that the stem cell antigen 1 (Sca-1) BM fraction is the subpopulation of stem cells that protects the aged heart (12). Sca-1 is a stem cell marker originally found on hematopoietic stem cells (13), but it is also present on cardiac progenitor cells in mice (14). Sca-1 has a role in hematopoiesis, and loss-of-function studies have shown increased fibrosis and cardiac hypertrophy with decreased cardiac function in an age-dependent manner (15–18). Sca-1 is also associated with the maintenance of the cardiac progenitor cell population (16, 18). This indicates that Sca-1 cells have an important role in heart function; however, how these multipotent cells affect the broader transcriptional cardiac milieu is unknown. Additionally, a lingering question has been, what role do BM-derived Sca-1 cells have on cardiac inflammation?

In this study, we investigated the cardiac-specific transcriptional changes associated with Sca-1 BM reconstitution in the context of aging and cardiac injury. Using young to old Sca-1^+ and Sca-1^- BM reconstitution and left coronary artery (LAD) ligation, we observed significant transcriptional and functional changes associated with inflammation and muscle health that correlate with improved heart function. At baseline (before injury), Sca-1^+ reconstitution induced the expression of genes associated with contractility and angiogenesis, which may prime the heart to adequately respond to cardiac injury. In addition, Sca-1^+ reconstitution reduced inflammatory gene expression at baseline but contributed to an efficient inflammatory response after MI. Thus, Sca-1 BM cells protect the heart *via* 2 gene programs.

MATERIALS AND METHODS

Experimental animals, cell preparation, and BM reconstitution

The Animal Care Committee of the University Health Network approved all experimental procedures, which were carried out according to the *Guide for the Care and Use of Laboratory Animals* (National Institutes of Health, Bethesda, MD, USA). Young donor BM was flushed from the tibiae and femurs of C57BL/6-Tg[CAG enhanced green fluorescent protein (GFP)]1Osb/J transgenic mice aged 2 to 3 mo (The Jackson Laboratory, Bar Harbor, ME, USA). Cells were dissociated in PBS using an 18- to 23-gauge needle. Cells were then incubated in 5 ml red blood cell lysis buffer (154.42 mM NH_4Cl, 11.9 mM $NaHCO_3$, 0.026 mM EDTA) for 5 min and then centrifuged for 5 min at 1000 rpm. These 2 steps were repeated for a total of 2 times each. The cell pellet was suspended in Iscove's Modified Dulbecco's Medium (Thermo Fisher Scientific, Waltham, MA, USA) and passed through a 40-μm filter to remove clumps. Cells were counted and separated into Sca-1 positively and negatively labeled fractions by immunomagnetic activated cell sorting following the manufacturer's instructions (Stemcell Technologies, Vancouver, BC, Canada). Female C57BL/6 mice aged 12 mo were lethally irradiated at dose of 9.5 Gy and immediately received an infusion (through the tail vein) of fresh Sca-1^+ or Sca-1^- BM cells (2×10^6) from young donor to generate Sca-1^+ or Sca-1^- chimeras. Three months later, flow cytometry ($n = 3$) was completed to determine the number of GFP^+ and Sca-1^+ [phycoerythrin (PE)-conjugated rat anti-mouse Sca-1; BD Biosciences, San Jose, CA, USA] donor cells in the blood and BM as previously described (2).

Myocardial infarction

Twelve weeks after BM reconstitution, coronary occlusion was performed as previously reported (2). Briefly, mice were intubated and ventilated with 2% isoflurane. Through a left thoracotomy, the LAD coronary artery was ligated. Three days after occlusion, the animals were euthanized under 5% isoflurane overdose, and hearts were collected for molecular and histologic analysis (n = 3–5 per group). The remaining mice ($n = 4$ per group) were used for cardiac functional analysis, which was performed by echocardiography at d 0 and 28 after MI.

Echocardiography

Mice were sedated with a 2% isoflurane (Pharmaceutical Partners of Canada, Richmond Hill, ON, Canada) nosecone. Echocardiographic examinations were performed with a GE Vivid 7 ultrasound system (GE Healthcare Canada, Mississauga, ON, Canada) with an i13L transducer. Depth and frequency were set at 1 cm and 14 MHz, respectively. Short-axis views were obtained from the parasternal approach. Left ventricular dimensions [left ventricular end-diastolic internal diameter (LVIDd) and end-systolic internal diameter (LVIDs)] were measured in M mode, and left ventricular areas [left ventricular end-diastolic internal area (LVIAd) and end-systolic internal area (LVIAs)] were measured in B mode at short-axis views of the left ventricle at midlevel. Ejection fraction was calculated as follows: [(LVIDd 3 − LVIDs 3)/LVIDd 3] × 100; and fractional area change was calculated as follows: [(LVIAd − LVIAs)/LVIAd] × 100.

Microarray analysis

The tissue was collected as follows: whole cardiac tissue was collected from reconstituted animals at baseline, whereas the scar and border zone were isolated from animals that received an MI. A total of 100 ng of RNA was prepared for quality analysis and subsequently prepared for microarray analysis using the MouseRef v2.0 BeadChip array (Illumina, San Diego, CA,USA) at the Princess Margaret Genomic Centre (Toronto, ON, Canada; *http://www.pmgenomics.ca/*). Data were analyzed by GeneSpring v13.0 and normalized by quantile normalization followed by a per-probe median centered normalization. Data were filtered such that only probes that were above the 20th percentile of the distribution of intensities in 100% of any of the 4 groups (Sca-$1^{-/+}$; Baseline/LAD3) were included in downstream analysis. Significantly upregulated genes were determined by 1-way ANOVA with a Benjamini-Hochberg false discovery rate (FDR) corrected $P <$ 0.05 and Tukey's *post hoc* test.

Bioinformatics

Heat maps and volcano plots were generated by R software (R Foundation for Statistical Computing, Vienna, Austria; *http://www.r-project.org/*). In the heat map analysis, if multiple probes for one gene were found to be differentially expressed, then gene names were labeled as Gene.1, Gene.2, *etc*. Gene Ontology (GO) analysis was completed using the Database for Annotation, Visualization, and Integrated Discovery (DAVID; *https://david.ncifcrf.gov/*). Predicted regulatory factors of differentially expressed genes were identified by oPOSSUM3.0 (*http://opossum.cisreg.ca/oPOSSUM3/*).

Immunofluorescence

Heart tissue was fixed in 2% paraformaldehyde overnight and placed in increasing sucrose solutions in succeeding nights. Tissue was analyzed for immunostaining with 5 to 10 μm sections. Sections were blocked with 5% bovine serum albumin. Primary antibodies were incubated for 1 to 2 h at room temperature in 5% bovine serum albumin. The following antibodies were used: GFP (A21311; Thermo Fisher Scientific); Mac-3 (BD Biosciences); F4/80 (Abcam, Cambridge, MA, USA); CD45 (BD Biosciences); and Sca-1 (R&D Systems, Minneapolis, MN, USA). Incubation with respective Alexa Fluor 546, 568, or 647 conjugated secondary antibodies (Thermo Fisher Scientific) was carried out at room temperature for 1 h. DAPI (Sigma-Aldrich, St. Louis, MO, USA) was used to stain nuclei. Cells were quantified manually using an average of 4 fields of view from 3 different mice per group. For experiments after MI, quantification was done using fields of view near the border zone. Cell quantification at baseline was done using 4 random fields.

Real-time quantitative PCR

RNA was isolated using Tri-Reagent (Sigma-Aldrich) according to the manufacturer's instructions. cDNA was prepared using NxGen M-MulV Reverse Transcriptase (Lucigen, Middletown, WI, USA) according to the manufacturer's instructions. cDNA expression was analyzed using SensiFast (Bioline, Taunton, MA, USA) SYBR Green using the following parameters: 95°C for 2 min (95°C for 5 s; 60°C for 30 s for 40 cycles). Primers are listed in Supplemental Table 1.

Statistical analysis

Statistical significance of quantitative PCR (qPCR) data and cell quantification was determined using an unpaired, 2-tailed Student's *t* test.

RESULTS

Preservation of heart function by young Sca-1$^+$ BM after injury

The cardiac-resident Sca-1 population has been shown to play a role in the preservation of heart function (16, 17), but the broad molecular changes invoked by Sca-1$^+$ BM reconstitution on the heart are unexplored. To understand the role of Sca-1$^+$ cells in heart function in the context of aging, 12-mo-old lethally irradiated mice were reconstituted with Sca-1$^-$ or Sca-1$^+$ BM cells from 3-mo-old GFP donor mice. Three months after reconstitution, stably reconstituted mice underwent ligation of the LAD to mimic MI, and mice were humanely killed 3 d later (referred to as LAD3) (**Fig. 1*A***). GFP$^+$ and/or Sca-1$^+$ BM and blood cells were quantified at baseline using flow cytometry (Fig. 1*B*). Sca-1$^+$ cells that were detected using flow cytometry are labeled as PE to avoid confusion with the reconstitution group labels. In the BM, cells positive for GFP and PE were significantly higher in the Sca-1$^+$ reconstituted group (Fig. 1*B*; $P < 0.05$). A small proportion (~2%) of all host-derived GFP$^-$ cells in the BM were PE$^+$ in either reconstitution group, indicating that host Sca-1$^+$ BM cells were effectively removed by irradiation. The majority of cells in the BM were host-derived GFP$^-$/PE$^-$ cells, which may represent nonhematopoietic cells that are less sensitive to irradiation. Significantly more GFP$^-$/PE$^-$ were detected in the Sca-1$^-$ reconstituted group ($P < 0.05$). A similar trend was seen in the blood; however, the percentage of GFP$^+$/PE$^+$ cells was considerably higher in the blood compared to the BM (50–60%).

Cross-sectional staining of the heart for GFP and CD45 showed recruitment of donor (GFP$^+$) and/or hematopoietic cells at baseline; however, the number of GFP$^+$ cells increased dramatically after MI, particularly in the Sca-1$^+$ group (Fig. 1*C, D*; $P < 0.05$). There were also significantly more GFP$^+$/CD45$^+$ cells in the heart after MI in the Sca-1$^+$ group compared to the Sca-1$^-$ reconstituted mice (Fig. 1*D*; $P < 0.05$). MI did not alter the number of GFP$^-$/CD45$^+$ (host) cells in the heart. Interestingly, GFP$^+$/CD45$^+$ cells represented a small proportion of the total amount of GFP cells present, indicating that Sca-1$^+$ BM contributes to other lineages. No GFP$^+$ cardiomyocytes were detected (data not shown). We examined ejection fraction and fractional area change at d 0 and 28 after LAD ligation (Fig. 1*E*). There were no differences in heart function at d 0 between Sca-1$^+$ and Sca-1$^-$ reconstituted mice; however, at d 28, Sca-1$^+$ reconstitution resulted in higher ejection fraction and fractional area change compared to Sca-1$^-$ controls, indicating a preservation of heart function after MI ($P < 0.05$).

Young Sca-1+ reconstitution of adult heart alters cardiac microenvironment

We hypothesized that the functional benefit observed 28 d after MI may be a consequence of molecular changes established by cardiac-resident GFP cells. To better understand the molecular changes induced by Sca-1$^+$ reconstitution, we completed a microarray analysis of Sca-1$^+$ and Sca-1$^-$ mice at baseline and LAD3 (scar and border zone). We initially examined the transcriptional changes associated with Sca-1$^+$ and Sca-1$^-$ reconstitution at baseline, which resulted in 275 differentially expressed probes. **Figure 2*A*** shows the spread of 275 differentially expressed probes in a volcano plot. Interestingly, we observed that genes associated with the calcium flux and muscle contraction—sarcolipin (*Sln*), myosin light chain 4 and 7 (*Myl4* and *Myl7*) and actin α1 (*Acta1*)—were the most drastically up-regulated. Factors expressed on the surface of T cells or associated with inflammation such as Cd3g and Cd274 were down-regulated. S100a8 was also down-regulated, but it was excluded from further analyses on the basis of Tukey's *post hoc* analysis.

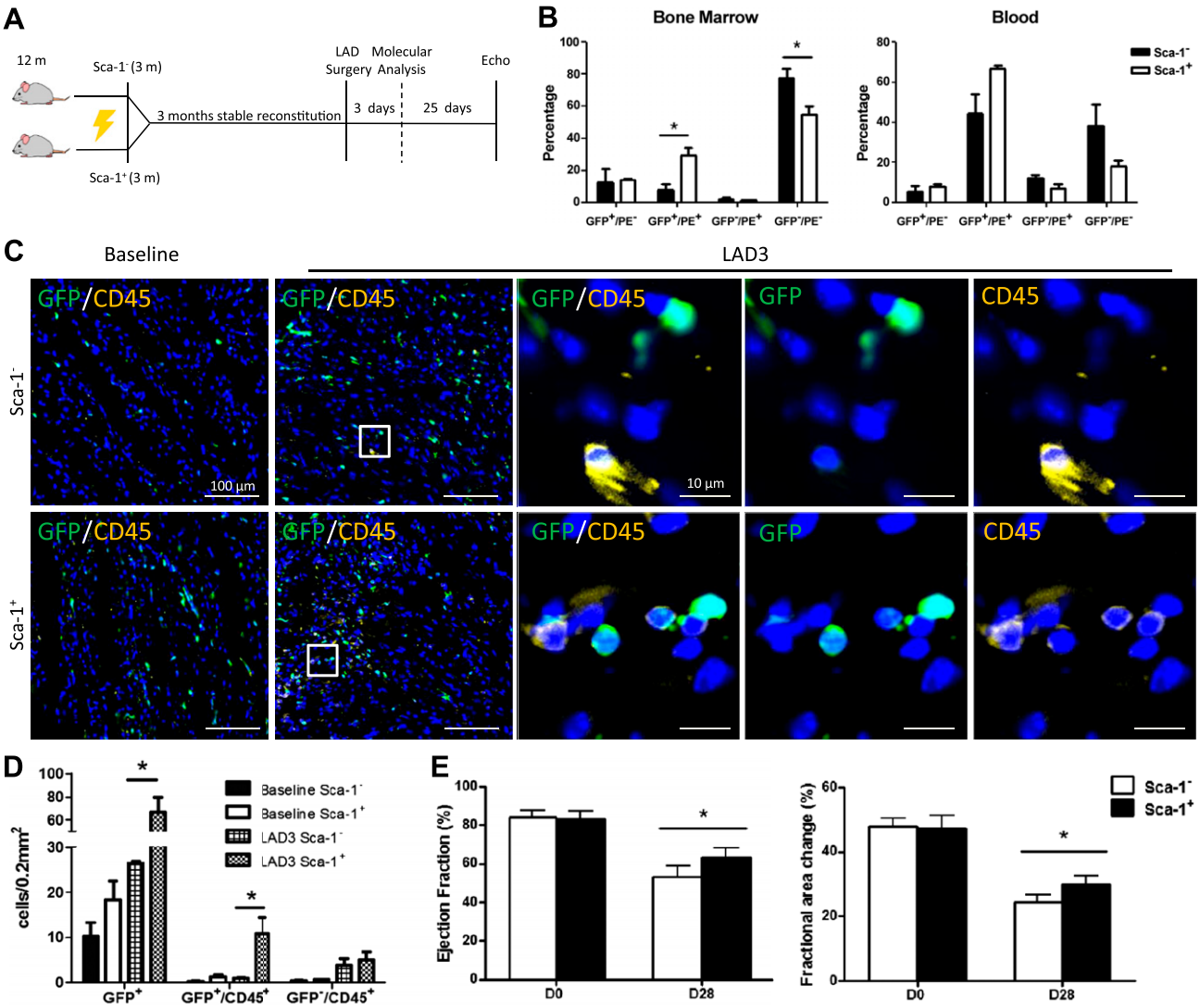

Figure 1. Effect of young Sca-1$^+$ BM reconstitution on heart function. *A*) Experimental design. BM of irradiated aged mice (12 mo) was reconstituted with 2×10^6 BM Sca-1$^+$ or Sca-1$^-$ cells from young GFP$^+$ donors (3 mo), generating Sca-1$^+$ chimeras [(YSca-1$^+$)-O] and Sca-1$^-$ chimeras [(YSca-1$^-$)-O] chimeras, respectively. Three months later, mice were humanely killed or treated with LAD ligation for 3 additional days (LAD3). *B*) Number of progenitor cells in chimeric mice after BM reconstitution. Twelve weeks later, GFP$^+$/Sca-1$^+$ cells in BM and blood of reconstituted mice were quantified by flow cytometry. For clarity, Sca-1$^+$ cells detected by flow cytometry are labeled as PE$^+$. Number of GFP$^+$/PE$^+$ cells in BM was significantly higher in (YSca-1$^+$)-O group compared to (YSca-1$^-$)-O group ($n = 3$). *C*) Homing of progenitor cells in chimeric hearts after BM reconstitution. Twelve weeks later, GFP$^+$/CD45$^+$ cells in heart at baseline and at 3 d after MI of reconstituted mice were detected. White and red boxes represent enlarged images shown at right. *D*) Quantitation of GFP$^+$ and/or CD45$^+$ cells in heart after Sca-1$^-$ or Sca-1$^+$ reconstitution ($n = 3$). *E*) Functional improvement of Sca-1$^+$ reconstituted mice 28 d after MI. Changes in ejection fraction and fractional area change were measured ($n = 4$). $^*P < 0.05$.

The biologic function of the differentially expressed genes was analyzed *via* GO analysis using the DAVID program. The 10 most enriched GO terms are shown in Supplemental Fig. 1; they included regulation of synaptic transmission, protein localization, actin cytoskeleton organization, and cellular calcium homeostasis. We expanded our search for genes involved in mesenchymal differentiation, contraction, and vascular development and generated a heat map to document the general trend of gene expression in these categories (Fig. 2*B*). In general, genes associated with sarcomere integrity, calcium flux, and muscle contraction were up-regulated. Transcriptional regulators enriched in cardiovascular tissues such as Jumonji domain-containing 6 (*Jmjd6*), nuclear receptor subfamily 2 group F, member 2 (*Nr2f2*), serum response factor (*SRF*), SET domain-containing 8 (*Setd8*), SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin, subfamily a, member 2 (*Smarca2*), and Ovo-like zinc finger 2 (*Ovol2*) were also modulated. We validated the expression patterns of *SRF*, *Jmjd6*, *Setd8*, and *Smarca2* using qPCR (Fig. 2*C*). Transcription factor *SRF* and *Jmjd6* (an arginine demethylase and lysine hydroxylase) are known to be involved in vascular development (19–21). *SRF* was significantly up-regulated ($P < 0.05$), but no significant difference in *Jmjd6* was found. *Smarca2* encodes brahma, which is a member of the SWI/SNF chromatin

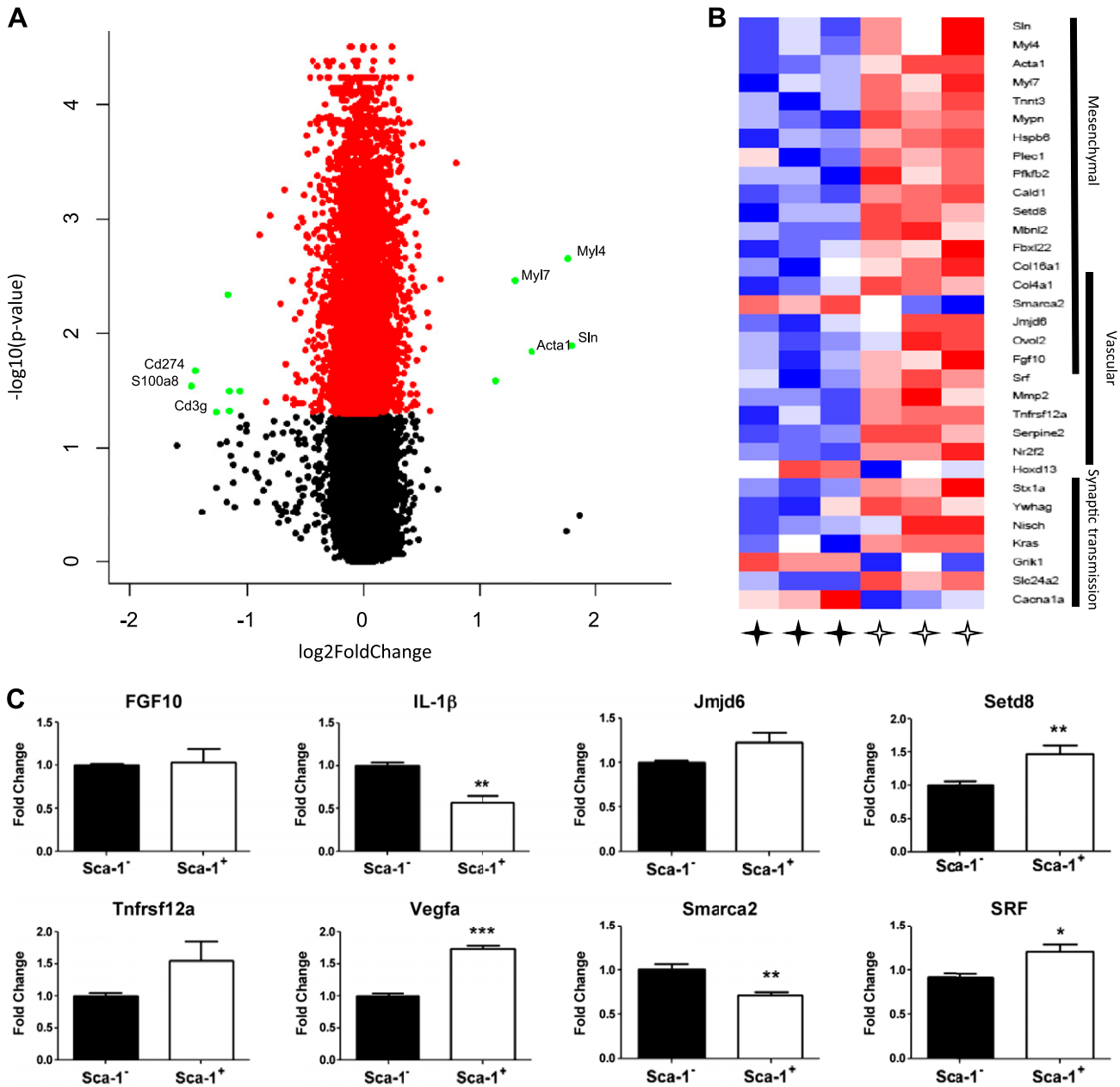

Figure 2. Changes in myocardial gene expression after young Sca-1$^+$ BM reconstitution. *A*) Volcano plot depicting $\log_2$ fold change of differentially expressed genes between Sca-1$^-$ and Sca-1$^+$ reconstituted mice at baseline. All 20,238 probes are represented here; *P* value is calculated based on 1-way ANOVA with Benjamini-Hochberg FDR correction. Data points are red if $P < 0.05$, green if $P < 0.05$, and $\log_2$FC $\geq$1.25. *B*) Heat map depicting microarray gene expression profiles of mesenchymal, vascular, and synaptic transmission related gene clusters. Black stars indicate Sca-1$^-$; white stars, Sca-1$^+$. *C*) Differential expression of mesenchymal, vascular, or cytokine related genes. Data were normalized to Gapdh and analyzed *via* $\Delta\Delta C_t$ method ($n = 5$). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

remodeling complex and regulates cell proliferation (22). Interestingly, *Smarca2* was down-regulated by Sca-1$^+$ reconstitution (Fig. 2*C*; $P < 0.01$). Last, *Setd8* expression was examined. *Setd8* is responsible for the H4K20me1 modification of histones, but it can also target p53 and may be recruited by Twist to promote epithelial-to-mesenchymal transitions (23–25). Like *SRF*, cardiac expression of *Setd8* was up-regulated with Sca-1$^+$ reconstitution (Fig. 2*C*; $P < 0.01$).

The expression of 4 secreted factors was also assessed using qPCR: TNF receptor superfamily, member 12a (*Tnfrsf12a*), fibroblast growth factor 10 (*FGF10*), vascular endothelial growth factor A (*Vegfa*), and *Il-1β*. Although *Vegfa* and *Il-1β* did not pass Tukey's *post hoc* analysis, we were curious about the expression of these genes, as we observed a +1.4- and -1.2-fold change in our microarray analysis, respectively (Supplemental Data Set 1). These 4 secreted factors have various roles in cardiomyocyte proliferation and death, angiogenesis, and the inflammatory response (26). *Vegfa* was up-regulated with Sca-1$^+$ reconstitution ($P < 0.001$), whereas Il-1 β was down-regulated, consistent with our microarray data (Fig. *2D;*

$P < 0.01$). *FGF10* and *Tnfrsf12a* showed no change in expression.

Sca-1$^+$ reconstitution induces dramatic transcriptional changes in injured heart

Differentially expressed genes were next assessed in Sca-1$^+$ and Sca-1$^-$ reconstituted mice at baseline and LAD3, corresponding to 5163 differentially expressed probes across all conditions (**Fig. 3*A***). Unbiased hierarchical clustering showed that LAD3 samples were distinct from baseline controls (Fig. 3*A*). Within LAD3 conditions, Sca-1$^+$ and Sca-1$^-$ gene expression profiles were clustered separately.

A total of 275, 3767, 4542, and 834 differentially expressed gene probes in Sca-1$^+$ *vs.* Sca-1$^-$ at baseline, Sca-1$^-$ LAD3 *vs.* Sca-1$^-$ baseline, Sca-1$^+$ LAD3 *vs.* Sca-1$^+$ baseline, and Sca-1$^+$ LAD3 *vs.* Sca-1$^-$ LAD3 were identified, respectively (Fig. 3*B*). The greatest number of differentially expressed genes was identified when comparing LAD3 to baseline in both Sca-1$^-$ and Sca-1$^+$ conditions.

Next we assessed the differential gene program at LAD3 between Sca-1$^+$ and Sca-1$^-$ reconstituted mice. We analyzed the biologic processes of these genes and found that within the 10 most up-regulated GO terms were clusters related to inflammation and apoptosis (Fig. 3*C*). A volcano plot was generated depicting all probes included in the microarray analysis based on the fold change calculated from the Sca-1$^-$ and Sca-1$^+$ groups after MI (Fig. 3*D*). The most strongly up-regulated genes such as *Chi3l1*, *Saa3*, *Ifit2*, *Ifit3*, *Ccl4*, and *Phf11* were related to inflammation.

Last, we compared putative transcriptional regulators of up- and down-regulated genes in our LAD3 Sca-1$^-$ and Sca-1$^+$ groups. Figure 3*E*, *F* shows the top 5 transcription factors predicted to regulate genes within 5 kb of the transcription start site of up-regulated or down-regulated genes, respectively. *Ctcf* is involved in gene regulation of multiple cell types, whereas the transcription factor families of *IRF*, *Nfil3*, and *Stat* are enriched in inflammatory cells and related processes (27–29). *Gabpa*, aka *NRF2*, is involved in metabolism and survival (30).

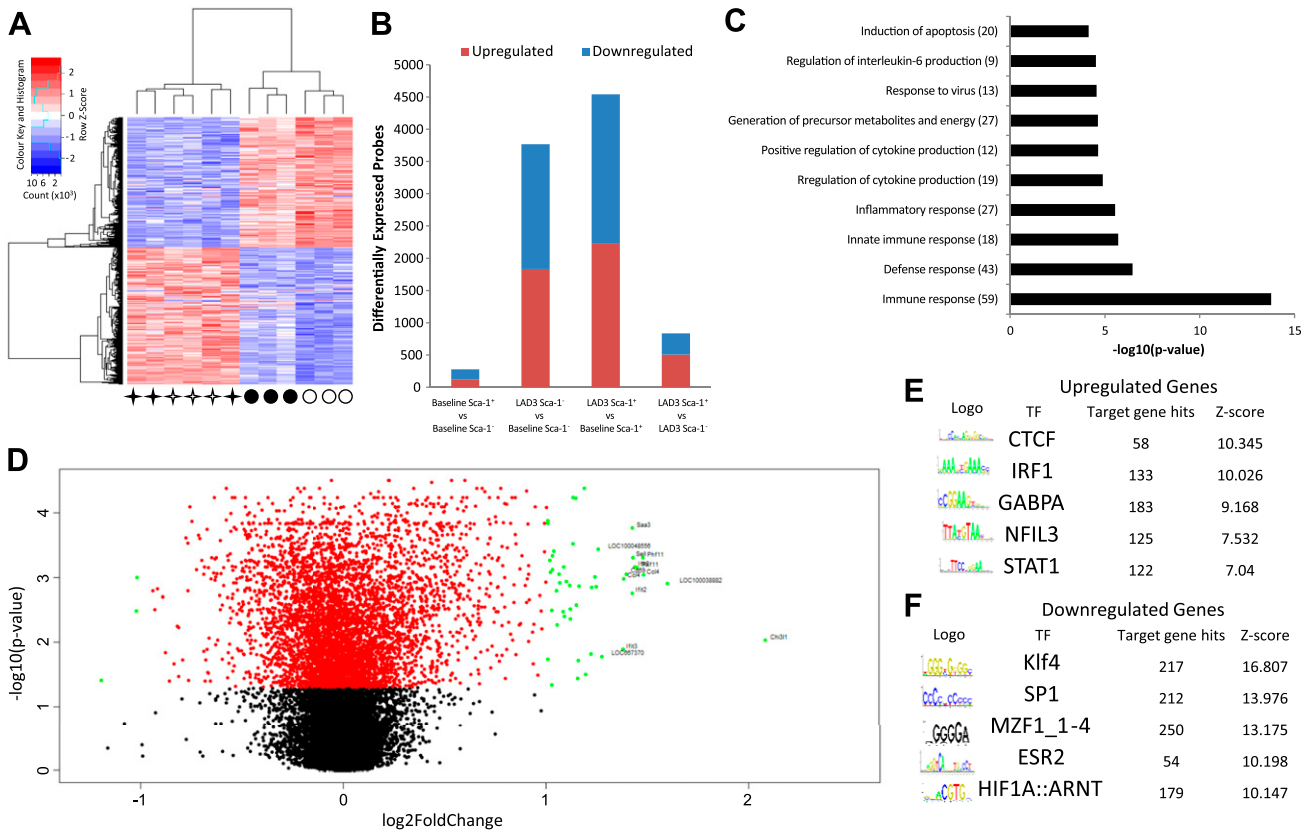

Figure 3. Young Sca-1$^+$ BM reconstitution alters gene expression within injured adult heart. *A*) Gene expression of scar and border zone were analyzed by microarray. Log_2-normalized data were analyzed by hierarchical clustering. Data represent 5163 remaining values across all conditions after 1-way ANOVA with Benjamini-Hochberg FDR corrected $P < 0.05$. Stars indicate baseline; circles, after ligation (MI); black, Sca-1$^-$; white, Sca-1$^+$. *B*) Total number of differentially expressed probes across all group comparisons. *C*) All up- and down-regulated probes ($n = 768$) from Sca-1$^+$ *vs.* Sca-1$^-$ samples after MI were pooled to identify GO terms for biologic processes associated with Sca-1$^+$ reconstitution after MI. Differentially expressed genes identified between Sca-1$^-$ and Sca-1$^+$ groups after MI were analyzed using DAVID. Top 10 biologic processes identified are included; number of genes associated with each term is in parentheses. *D*) Volcano plot depicting log_2 fold change of differentially expressed genes between Sca-1$^-$ and Sca-1$^+$ reconstituted mice after MI. All 20,238 probes are represented; *P* value is calculated based on 1-way ANOVA with Benjamini-Hochberg FDR correction. Data points are red if $P < 0.05$, green if $P < 0.05$, and log_2FC ≥ 1. Total of 5163 probes have $P < 0.05$. *E*, *F*) Predicted regulatory factors of up- and down-regulated genes that were identified in *C* (Sca-1$^+$ after MI *vs.* Sca-1$^-$ after MI). Top 5 predicted regulatory factors are shown based on their *z* score as calculated by oPOSSUM3.0.

Down-regulated genes were predicted targets for HIF1α (HIF1A:ARNT) (Fig. 3*F*). *KLF4* and *MZF* are members of the Kruppel family of zinc finger transcription factors, and *Sp1* is a closely related transcription factor that has been implicated in cardiac hypertrophy (31). These predicted transcriptional regulators corroborate our GO analysis, which showed up-regulation of inflammatory related gene programs by Sca-1$^+$ reconstitution.

Inflammatory response is up-regulated in Sca-1$^+$ reconstituted mice

Many of the up-regulated genes were associated with inflammation, including secreted factors or components involved in inflammatory cell pathways. **Figure 4*A*** depicts recruitment of GFP$^+$ cells across an entire section from Sca-1$^-$ or Sca-1$^+$ reconstituted mouse hearts. GFP$^+$ cells were localized to the scar and border in both groups compared to the remote area (Fig. 4*A*). At baseline, reconstitution resulted in fewer cardiac-resident GFP$^+$ cells compared to LAD3, which were localized throughout the heart nonspecifically (Supplemental Fig. S3). Therefore, post-MI donor BM cells reside near the site of injury. We next divided all inflammatory-related genes into inflammation-related factors or inflammation-related receptors to visualize the expression pattern across all groups in heat maps (Fig. 4*B*, *C*). The up-regulation of several cytokines such as *Ccl2* (MCP-1), *Ccl3*, and *Ccl4* suggests the inflammatory cells were recruited and activated after Sca-1$^+$, but not Sca-1$^-$, reconstitution (Fig. 4*B*).

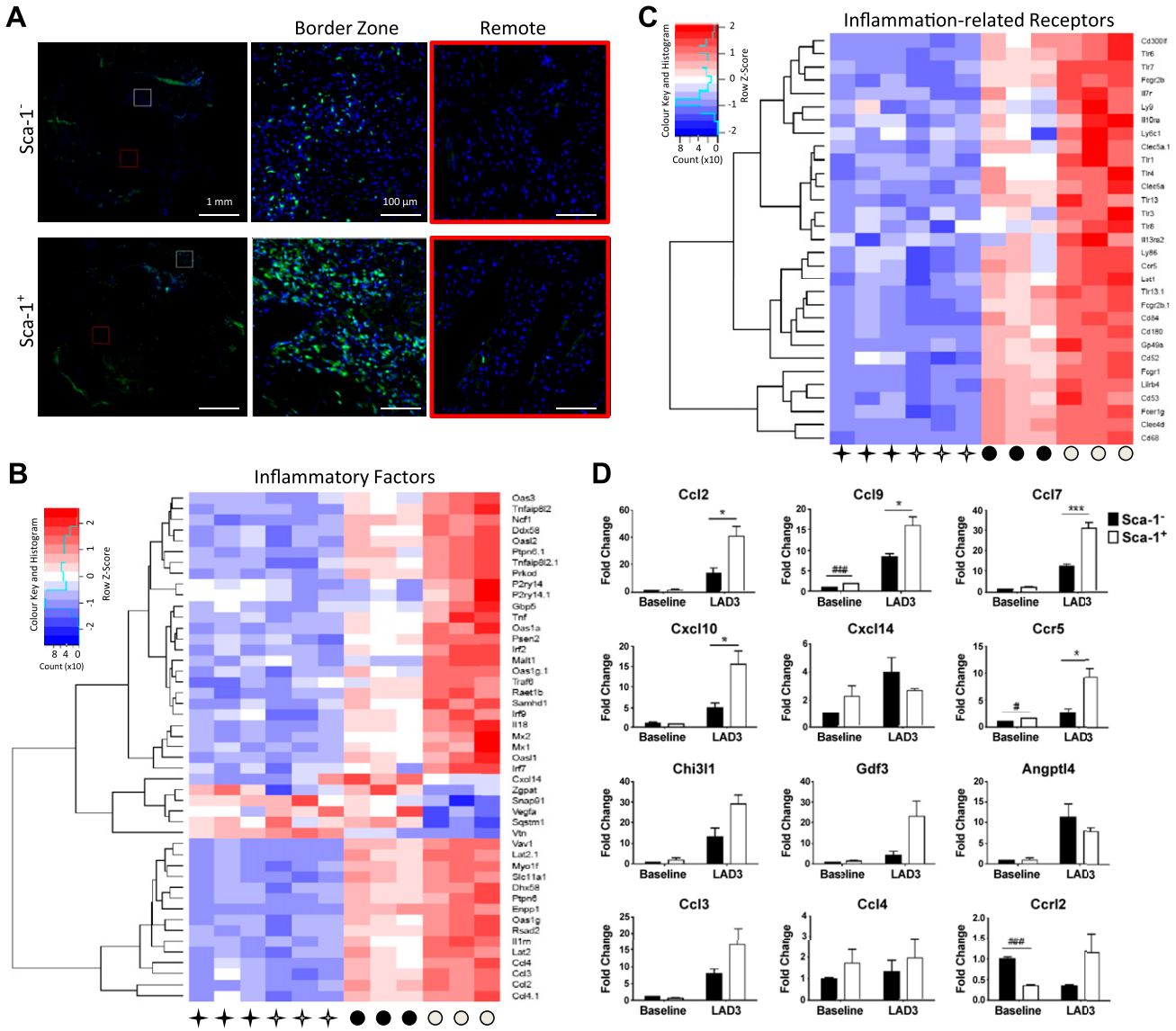

Figure 4. Sca-1$^+$ reconstitution induced expression of inflammatory markers in heart after injury. *A*) Recruitment of GFP$^+$ cells in Sca-1$^+$ or Sca-1$^-$ reconstituted mice after MI in border zone and remote areas of heart. White and red boxes represent enlarged images shown at right and represent border zone and remote area, respectively. *B*, *C*) Microarray expression of genes identified as inflammatory factors (secreted proteins or transcription factors) (*B*) or inflammation-related receptors (*C*) are depicted in heat map. Stars indicate baseline; circles, after ligation (MI); black, Sca-1$^-$; white, Sca-1$^+$. *D*) Validation of changes in inflammation-related genes *via* qPCR. Data were normalized to Gapdh and analyzed *via* $\Delta\Delta C_t$ method ($n = 3$). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ between Sca-1$^-$ LAD3 and Sca-1$^+$ LAD3; $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$ between Sca-1$^+$ and Sca-1$^-$ at baseline.

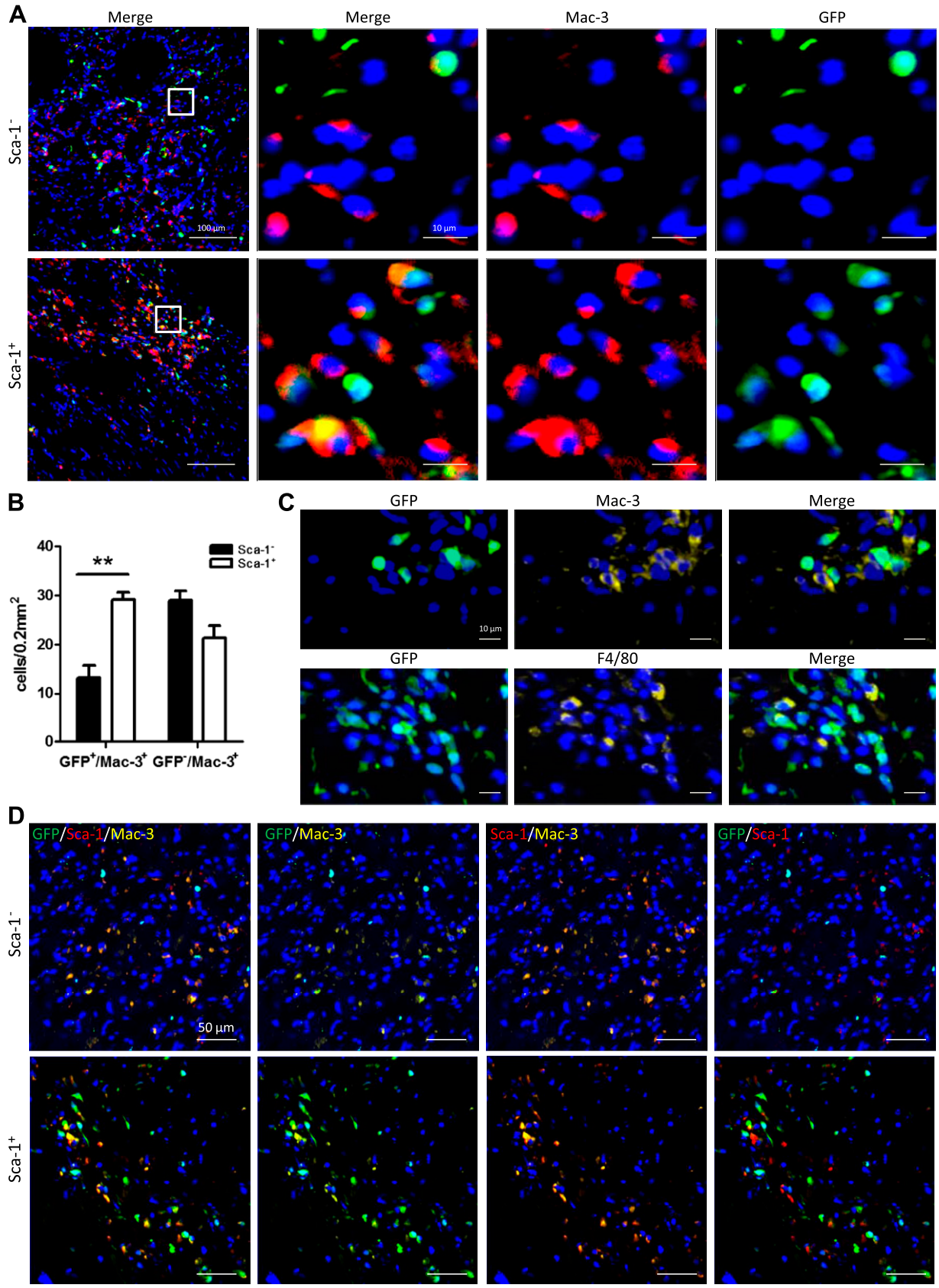

Figure 5. Differential activation of host or donor macrophages in aged heart. *A*) Mac-3 is expressed in both donor and host cardiac resident cells after MI. After reconstitution, mice underwent LAD coronary artery ligation and were humanely killed 3 d later. Immunofluorescent staining for GFP^+ (donor cells) and macrophages (Mac-3; red) was performed. White box represents enlarged image shown at right. *B*) Quantification of donor- and host-derived macrophages. There are significantly more donor

(*continued on next page*)

Indeed, several cell surface markers found on immune cells were up-regulated, including macrophage markers Cd68 and Ly6c1 (Fig. 4*C*). Seven Toll-like receptors and *Irf* transcription factors were up-regulated as well (Fig. 4*B, C*).

We validated the expression levels of various secreted factors related to inflammation, including *Ccl2, Ccl3, Ccl4, Ccl7,* and *Ccl9* and cytokine receptors *Ccrl2* and *Ccr5* (Fig. 4*D*). *Ccl7*, *Ccl9*, and *Cxcl10* had been initially excluded after Tukey's *post hoc* analysis, but were up-regulated 1.6-, 1.3-, and 2.1-fold in the microarray analysis, respectively (Supplemental Data Set 1). Figure 4*D* confirmed the trend of our microarray data and demonstrates an up-regulation of cytokine production and cytokine receptor expression in the Sca-1$^+$ reconstituted mice after MI. These data also reveal that at baseline, the inflammatory response is altered as *Ccl9* and *Ccr5* were significantly up-regulated ($P < 0.001$; $P < 0.05$), while *Ccrl2* was significantly down-regulated (Fig. 4*D*; $P < 0.001$). The expression of several other secreted molecules (*Cxcl10, Cxcl14, Chi3l1, Angptl4, and Gdf3*) was also assessed using qPCR (Fig. 4*D*).

Apoptosis was identified as an enriched category in GO term analysis (Fig. 3C). To visualize changes in cell death, we generated a heat map of the corresponding genes (Supplemental Fig. 2). Although genes associated with canonical cell death pathways were differentially expressed (*e.g., Bid, Apaf1, Casp9*) we noticed that a core group of genes are related to the inflammasome, which is classically involved in macrophage-mediated cell death after bacterial infection (32), were up-regulated (Supplemental Fig. 2). These included *Casp1, Casp4,* and *Pycard. Casp1* is also responsible for cleavage of *Il-1β* and Il-18, the latter of which was up-regulated in Sca-1$^+$ reconstitution (Fig. 4*B*).

Sca-1$^+$ BM contributes to cardiac macrophage population

Because there was considerable change in the inflammatory gene program with Sca-1$^+$ reconstitution after MI, we next assessed the contribution of donor BM to the cardiac macrophage population. To test this, we costained for GFP (donor cells) and Mac-3. After MI, Sca-1$^+$ reconstituted BM contributed to significantly more cardiac macrophages compared to Sca-1$^-$ (**Fig. 5*A, B***; $P < 0.01$). The number of GFP$^-$/Mac-3$^+$ cells was not affected by Sca-1$^+$ reconstitution, demonstrating that there is a mix of host and donor Mac-3$^+$ cells in the heart.

We repeated this experiment using older animals that were reconstituted at 18 to 20 mo old (21–23 mo at time of death), as this age group better reflects biologic phenomena within the aged population. Three days after MI, a portion of young GFP$^+$ donor cells was positive for Mac-3 or F4/80 in cardiac tissue from the Sca-1$^+$ reconstituted mice. Some GFP$^+$ cells were Mac-3$^-$ and F4/80$^-$, indicating that GFP$^+$ cells can contribute to different cell lineages (Fig. 5C). The Mac-3$^+$/GFP$^-$ or F4/80$^+$/GFP$^-$ cells are host-derived inflammatory cells.

To determine whether GFP$^+$ donor cells retain Sca-1 expression after they home to the heart, we next stained for Sca-1 in reconstituted aged mice (>18 mo) 3 d after MI. We also costained for Mac-3 to identify inflammatory cells. Sca-1$^-$ reconstitution resulted in fewer cardiac GFP$^+$ cells overall, and none was positive for Sca-1 or Mac-3 expression (Fig. 5*D*). All donor-derived Mac-3$^+$ cells, however, coexpressed Sca-1. Mice that that were reconstituted with Sca-1$^+$ BM contributed GFP$^+$ cells to the Mac-3$^+$/Sca-1$^+$ population (triple positive), but 2 additional populations of cells were also present: Mac-3$^+$/Sca-1$^+$/GFP$^-$ and Mac-3$^-$/Sca-1$^-$/GFP$^+$. Thus, Sca-1$^+$ donor BM cells lose Sca-1 expression if they do not contribute to the inflammatory cell population, resulting in a trifecta of noninflammatory donor cells (Mac-3$^-$/Sca-1$^-$/GFP$^+$), inflammatory donor cells (Mac-3$^+$/Sca-1$^+$/GFP$^+$), and inflammatory host cells (Mac-3$^+$/Sca-1$^+$/GFP$^-$) to be present in the reconstituted mouse heart after MI.

DISCUSSION

We have identified some of the molecular alterations associated with cardioprotection with Sca-1$^+$ BM cells. Initially, Sca-1$^+$ BM cells migrate and reside in cardiac tissue to provide the potential for efficient recovery after injury *via* up-regulation of vascular and contractile-related genes while mitigating inflammatory gene expression such as *Il-1β* (Fig. 2). Three days after cardiac injury (MI), Sca-1$^+$ cells are responsible for the inflammatory response (Fig. 4). Together, the combined effects of these 2 independent programs of BM-derived Sca-1$^+$ cells contribute to efficient healing, first by priming the heart for protection from injury *via* the up-regulation of growth factors (*e.g., Vegfa*), and second by producing an effective inflammatory response that is necessary for cardiac recovery. This limits overall loss of cardiac function (Fig. 1).

Harnessing the reparative power of circulating progenitor cells in cell therapy has remained elusive because of their molecular complexity. Failure of circulating progenitor cells to become activated, home to the heart, and differentiate is a significant limitation of cardiovascular cell therapy associated with aging (33, 34). This intricacy has been supported by a human study that showed that a combination of age and risk factors leads to a reduction in circulating progenitor cells (3). BM reconstitution provides the theoretical framework for understanding circulating progenitor cells in the context of disease, such as MI and aging, as shown here. Since *in vivo* differentiation of BM-derived progenitor cells to endothelial cells and cardiomyocytes in animal models is possible (10), it is of

macrophages (GFP$^+$/Mac-3$^+$) cells in Sca-1$^+$ reconstituted mice ($n = 3$). **$P < 0.01$. *C*) Aged mice (>18 mo) were reconstituted with young Sca-1$^+$ BM and underwent MI for 3 d as described in Fig. 1*A*. Macrophages were detected using Mac-3 or F4/80 and costained for GFP to demonstrate contribution of donor BM cells to cardiac macrophage population. *D*) Heart sections from aged mice (>18 mo) reconstituted with Sca-1$^+$ or Sca-1$^-$ BM were next stained for Sca-1 (red) in combination with GFP and Mac-3 (yellow) after MI.

interest to understand the molecular programs driven by these cells to optimize the translational benefits in humans. Dey *et al.* (35) completed microarray analysis on BM-derived c-Kit$^+$ cells, BM-derived stem cells, cardiomyocytes, cardiac-resident Sca-1$^+$ cells, and side population cells and determined that BM-derived c-Kit$^+$ cells were the most primitive and distinct from cardiac lineages, whereas Sca-1$^+$ cardiac resident cells were most similar to cardiomyocytes. The similarity between cardiac-resident Sca-1$^+$ cells and cardiomyocytes was strengthened using single-cell RNA profiling (36). Although we observed Sca-1$^+$ BM cells to play a predominately supportive role in endogenous repair mechanisms in the heart at baseline, Liu *et al.* (11) have proposed that BM cells also contribute to the cardiac progenitor pool, and thus the distinction between BM-derived cells and cardiac resident stem cells requires further study.

Our analysis identified some of the transcriptional changes that occur as a consequence of young Sca-1$^+$ BM reconstitution in adult mice. At baseline, Sca-1$^+$ reconstitution modifies the expression of several key transcriptional regulators, including *SRF, Smarca2,* and *Setd8.* Changes in these genes could indicate a transcriptional landscape that is proactive to angiogenic signals, thus preemptively preparing the heart to stress. Chromatin accessibility and histone modifications change with aging and thus may have an important role in stem cell activation in the hear (37). A more precise examination of the chromatin changes induced by cell therapy on resident progenitor cells would identify these consequences. Additionally, Sca-1$^+$ reconstitution reduced expression of *Il-1β* and cell surface markers associated with inflammatory cells (Fig. 2). This could indicate that young Sca-1$^+$ cells reduce chronic cardiac inflammation, which progresses with age. In this vein, Sca-1$^+$ reconstitution showed that its most robust effect after MI is to regulate inflammation, yet functionally, the heart was protected 28 d after MI (Fig. 1). The importance of inflammation in cardiac repair is considered to be a delicate balance, where chronic inflammation is maladaptive yet an efficient inflammatory response is also necessary for wound repair and healing (38, 39). After injury in the adult heart, the population of healing cardiac-resident macrophages becomes replaced by monocyte-derived macrophages that drive inflammation (40). As irradiation removed all Sca-1$^+$ host cells (GFP$^-$/PE$^+$) in the BM, the hematopoietic niche was replaced with young Sca-1$^+$ donor cells (Fig. 1*B*). Sca-1$^-$ cells, however, could not reconstitute the BM as efficiently. This corresponded to increased levels of donor CD45$^+$ cells and macrophages in the heart after MI; however, an equal amount of host CD45$^+$/GFP$^-$ and Mac-3$^+$/GFP$^-$ cells were also present (Fig. 1*D* and Fig. 5). We have previously shown that it is the heart-resident BM cells that are required for heart repair using a microchimerism model (2). In this light, we propose that although Sca-1 cells can contribute to the macrophage population, it is the initial cells that reside in the heart before MI that induce activation of inflammatory pathways *via* cytokine expression. One possibility is that Sca-1 BM cells shape the cardiac milieu by secreting factors that first direct timely inflammation and then initiate angiogenesis. Interestingly, *Irf7,* a master of the M1 to M2 transition (41), which corresponds to inflammatory and healing macrophages, respectively, was also up-regulated by Sca-1$^+$ reconstitution. Thus, Sca-1 progenitor cells could mediate the rate of the M1–M2 transition. Alternatively, the most beneficial Sca-1 subtype could be from alternative lineages. Notably, a number of GFP$^+$PE$^-$ cells were also present in the BM (Fig. 1*B*), which suggests the presence of nonhematopoietic donor progenitors, such as endothelial progenitor cells. This likelihood is also reflected by the low abundance of CD45$^+$/GFP$^+$ cells after MI relative to the total number of GFP cells (Fig. 1*C, D*).

CONCLUSIONS

These data demonstrate the cardiac-specific transcriptional changes associated with young Sca-1$^+$ BM reconstitution in adult mice. Transcriptional changes in the adult myocardium induced by Sca-1$^+$ BM cells may limit chronic inflammation and prime the heart to efficiently respond to injury, which has important implications in our understanding of age-associated cardiac inflammation. FJ

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AUTHOR CONTRIBUTIONS

S. W. Tobin performed research, analyzed data, assembled the figures, and wrote the article; S.-H. Li, R.-K. Li, and R. D. Weisel designed the experiment; and S.-H. Li, J. Li, J. Wu, A. Yeganeh, and P. Yu performed research and analyzed data.

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