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Short Communication

Epicardium is required for cardiac seeding by yolk sac macrophages, precursors of resident macrophages of the adult heart

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ABSTRACT

A subset of macrophages that reside in adult tissues originate from the fetal yolk sac, while others derive from circulating monocytes. These ontologically different macrophage subsets have distinct roles in tissue injury responses, with the embryonic population overall having beneficial activity in cardiac repair. Here we show that fetal yolk macrophages are recruited to a niche within and just below the epicardium, the mesothelial covering of the heart. The epicardium was required for establishment of yolk sac macrophages in this region of the fetal heart, and this function of epicardium depended on its expression of the transcription factor WT1. Thus, tissue-specific cues and transcriptional programs recruit or retain embryonic macrophages in their final abodes, where they help to shape organ homeostasis and injury responses.

1. Introduction

Macrophages are a heterogeneous population of phagocytic cells that contribute to a wide range of developmental, homeostatic, and inflammatory processes (Davies et al., 2013). There has been increasing awareness that macrophage heterogeneity also extends to their developmental origins (Epelman et al., 2014a,b). Recent lineage tracing studies have revealed that a subset of resident tissue macrophages of the adult heart, liver, and brain originate from the fetal yolk sac and persist separately from the blood monocyte pool (Schulz et al., 2012; Epelman et al., 2014a,b). These resident macrophages, derived from fetal yolk sac macrophages and characterized by developmental expression of the receptor CSF1R, have distinct functional properties from adult blood monocyte-derived tissue macrophages (Epelman et al., 2014a; Lavine et al., 2014; Epelman et al., 2014b).

In the heart, macrophages have both inflammatory and reparative roles (Weinberger and Schulz, 2015; Aurora et al., 2014; Lavine et al., 2014). These dichotomous functions may relate to ontologically distinct macrophage subsets, with adult blood monocyte-derived macrophages, marked by expression of the receptor CCR2, favoring inflammatory responses and embryonically-derived macrophages (CSF1R lineage; CCR2 negative) participating more in tissue repair and regeneration (Epelman et al., 2014a; Lavine et al., 2014). Embryonic homing of macrophages to the fetal heart has also been implicated in cardiac lymphatic development (Klotz et al., 2015). While the importance of embryonically-derived macrophages is clear, the mechanisms by which they are recruited to and retained in the heart from their extra-cardiac origins such as the fetal yolk sac (Schulz et al., 2012) are unknown.

In this study we report that fetal yolk sac macrophages colonize a niche within and just below the epicardium, the mesothelium that covers the outer surface of the heart. The epicardium was required for establishment of fetal yolk sac macrophages in the heart. Ablation of the epicardium, or mutation of the epicardium-restricted transcription factor Wilm's tumor gene 1 (WT1), impeded fetal yolk sac macrophage establishment in the heart. Our data suggest that specific paracrine signals or intercellular communications are responsible for recruitment or retention of the embryonic macrophages in the locales that they will patrol as adult tissue-resident macrophage.

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2. Results

2.1. The majority of early epicardial LYVE1$^+$ cells are macrophages

In order to better characterize macrophages entering the heart during development, we stained fetal heart sections using macrophage markers. One well established marker for cells of the monocyte/macrophage lineage is CD68 (Micklem et al., 1989). LYVE1 has also been reported to mark blood lineages including macrophages (Flaht et al., 2012), in addition to marking developing cardiac lymphatics (Flaht-Zabost et al., 2014). At E14.5, CD68$^+$ and LYVE1$^+$ cells were concentrated either within or adjacent to the epicardium, the epithelial layer that covers the surface of the heart (Fig. 1A and Suppl. Fig. 1). Quantitative analysis of co-expression of CD68 and LYVE1 showed that most cells in this region were predominantly positive for both markers (Fig. 1C). Since LYVE1 also marks lymphatic endothelial cells, we analyzed the co-expression of LYVE1 and endothelial cell marker CD31. At E14.5, few LYVE1$^+$ cells co-expressed CD31 (8%; Fig. 1B and D). These data indicate that at E14.5, LYVE1 and CD68 largely mark macrophages in the heart.

By E16.5, there is marked expansion of the cardiac lymphatic endothelial cells (CD68$^-$ LYVE1$^+$ CD31$^+$) in the subepicardial and subendocardial regions (Klotz et al., 2015). As a result, most LYVE1$^+$ cells no longer co-expressed the macrophage marker CD68 and rather co-expressed the endothelial cell marker CD31 (Fig. 1C and D and Suppl. Fig. 2). Consistent with the expansion of lymphatic endothelial cells, LYVE1$^+$ CD68$^+$ cells made up a smaller fraction of all marked cells at E16.5 (Fig. 1C). These cells remained localized in the epicardial region (Suppl. Fig. 2).

We also examined the distribution of macrophages in the heart at two earlier time points, E11.5 and E13.5 (Suppl. Fig. 3). Like macrophages, coronary vasculature is also intimately associated with epicardium. The developing vascular plexus is first observed on the dorsal surface of the heart near the AV groove. It then spreads apically and wraps around to the ventral surface of the heart. Co-staining of coronary vessel (CD31) and macrophage (CD68) markers showed that macrophages are located in close proximity with coronary endothelium and epicardium, and populate the heart in the same base-to-apex, dorsal-to-ventral pattern. Like the coronary vascular plexus, atrial occupancy by macrophages was completed before the ventricles, but by E14.5 the atria and ventricles and left and right heart became equally populated. Since coronary blood flow is not established until E14.5, the close relationship between macrophages and endothelial cells likely reflects mutually supportive paracrine signaling.

In order to further characterize the LYVE1$^+$ cells at E14.5, we isolated them by flow cytometry in conjunction with CD31 and a panel of blood/macrophage markers (CD45, CD11b, and F4/80, referred to collectively as BMAC; Fig. 1E). We used qRTPCR to

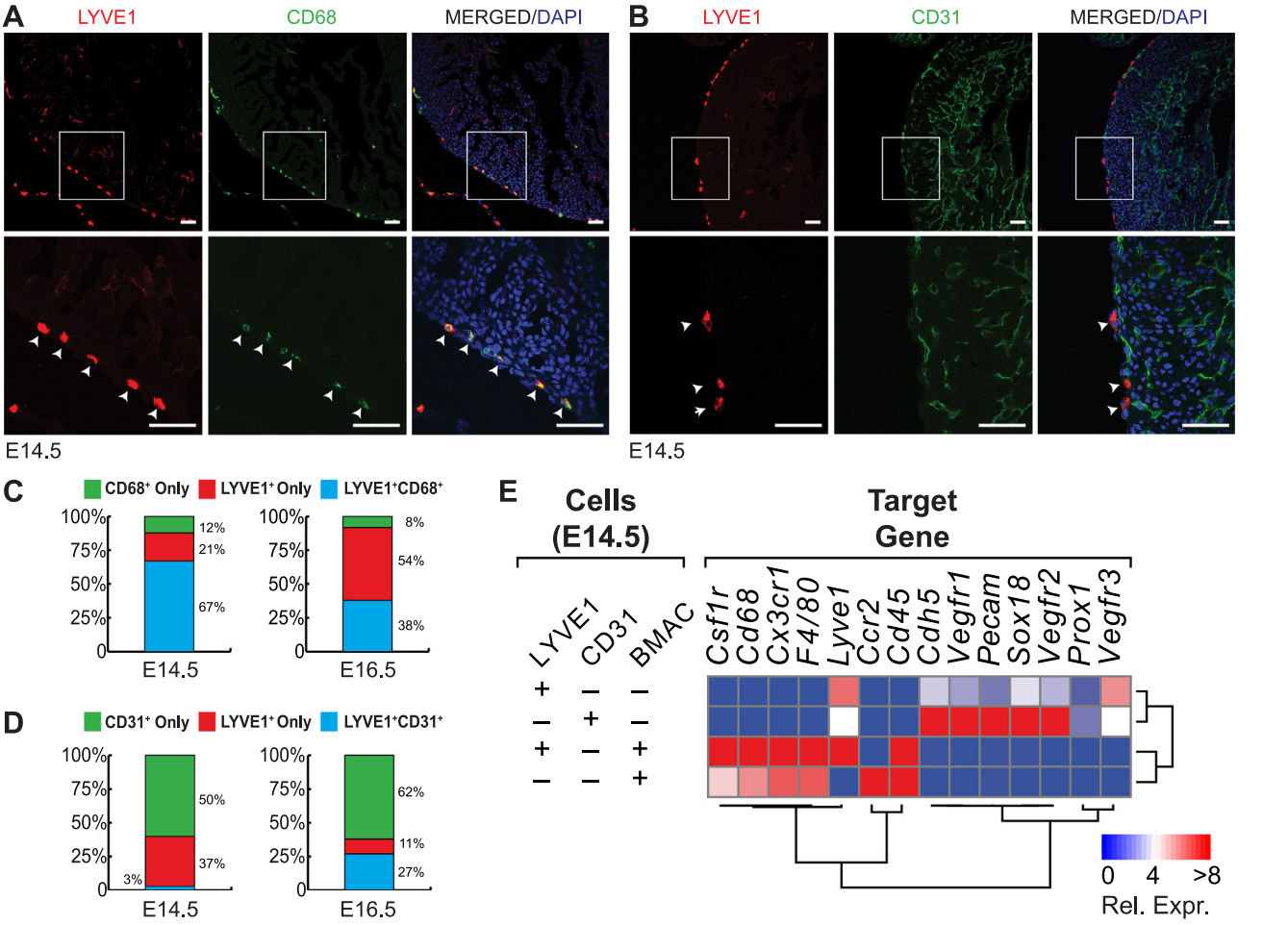

Fig. 1. Characterization of LYVE1$^+$ CD68$^+$ cells in the fetal heart. (A–B) E14.5 heart cryosections immunostained for expression of LYVE1 and CD68 or LYVE1 and CD31. Boxed regions of the upper row are magnified in the lower row. Arrowheads indicate cells co-expressing the stained antigens. Note that the co-expressing cells localized to the epicardial region. Bar = 50 μm. (C–D) Fraction of cells expressing either one or both stained antigen in the subepicardial region. $n = 3$ embryos per stage. (E) E14.5 fetal hearts were dissociated and FACS sorted based on their expression of LYVE1, CD31, and a panel of blood and macrophage markers (BMAC = CD45 CD11b F4/80). Gene expression was measured in RNA isolated from the indicated cell populations and expressed as fold-change compared to the input, unsorted cells. Genes with undetectable expression in a sorted population were assigned a value of zero.

measure the expression of several blood cell, endothelial cell, and macrophage markers in the sorted cell populations. The LYVE1$^+$ CD31$^-$ BMAC$^+$ and LYVE1$^+$ CD31$^-$ BMAC$^-$ cells were enriched for LYVE1, whereas the LYVE1$^-$ CD31$^-$ BMAC$^+$ cells were not, confirming the effectiveness of the sorting. The LYVE1$^+$ CD31$^-$ BMAC$^+$ cells were enriched for macrophage markers including *Cd68*, *Cx3cr1*, F4/80, and *Csf1r*, even more so than the LYVE1$^-$ BMAC$^+$ population (Fig. 1E), indicating that the LYVE1$^+$ BMAC$^+$ population represents macrophages.

Ccr2 encodes a receptor that is differentially expressed on resident postnatal macrophages derived from the circulation (CCR2$^+$) compared to those derived from the embryo (CCR2$^-$) (Epelman et al., 2014b; Lavine et al., 2014). The expression of *Ccr2* in our FACS-sorted populations from E14.5 heart indicated that the LYVE1$^+$ BMAC$^+$ cells represent a specific subset of embryonic macrophages: LYVE1$^+$ BMAC$^+$ cells lacked *Ccr2*, whereas LYVE1$^-$ BMAC$^+$ cells strongly expressed *Ccr2* (Fig. 1E). Together our data, integrated with the literature, indicate that cardiac LYVE1$^+$ macrophages at E14.5 are largely a CCR2$^-$ subset of embryonic macrophages that are recruited to the fetal heart and give rise to resident macrophages of the adult heart (Epelman et al., 2014a).

The BMAC$^-$ and BMAC$^+$ subsets of LYVE1$^+$ CD31$^-$ cells had distinct expression patterns. The BMAC$^-$ subset did not express blood or macrophage markers but were enriched for endothelial cell markers. Compared to endothelial cells (LYVE1$^-$ CD31$^+$ BMAC$^-$), the LYVE1$^+$ CD31$^-$ BMAC$^-$ population was less enriched for common endothelial cell markers (*Cd31*, *Cdh5*, *Vegfr1, Vegfr2*, *Sox18*) and relatively more enriched for the lymphatic endothelial cell marker *Vegfr3*. However, these cells expressed low levels of the key lymphatic transcription factor *Prox1*. These data suggest that these E14.5 LYVE1$^+$ CD31$^-$ BMAC$^-$ cells, which comprise a small fraction of all LYVE1$^+$ cells at this stage, represent a nascent lymphatic endothelial cell lineage.

Together these data indicate that at E14.5 LYVE1 and CD68 largely co-mark a subset of embryonic macrophages that accumulate in the epicardial region. These macrophages compose most LYVE1$^+$ cells at this stage. However, the LYVE1$^+$ CD31$^+$ CD68$^-$ lymphatic endothelial cell population expands rapidly, so that by E16.5 they compose most of the LYVE1$^+$ cells in the heart. The LYVE1$^+$ CD68$^+$ macrophage population persists at this stage, particularly within the epicardial region.

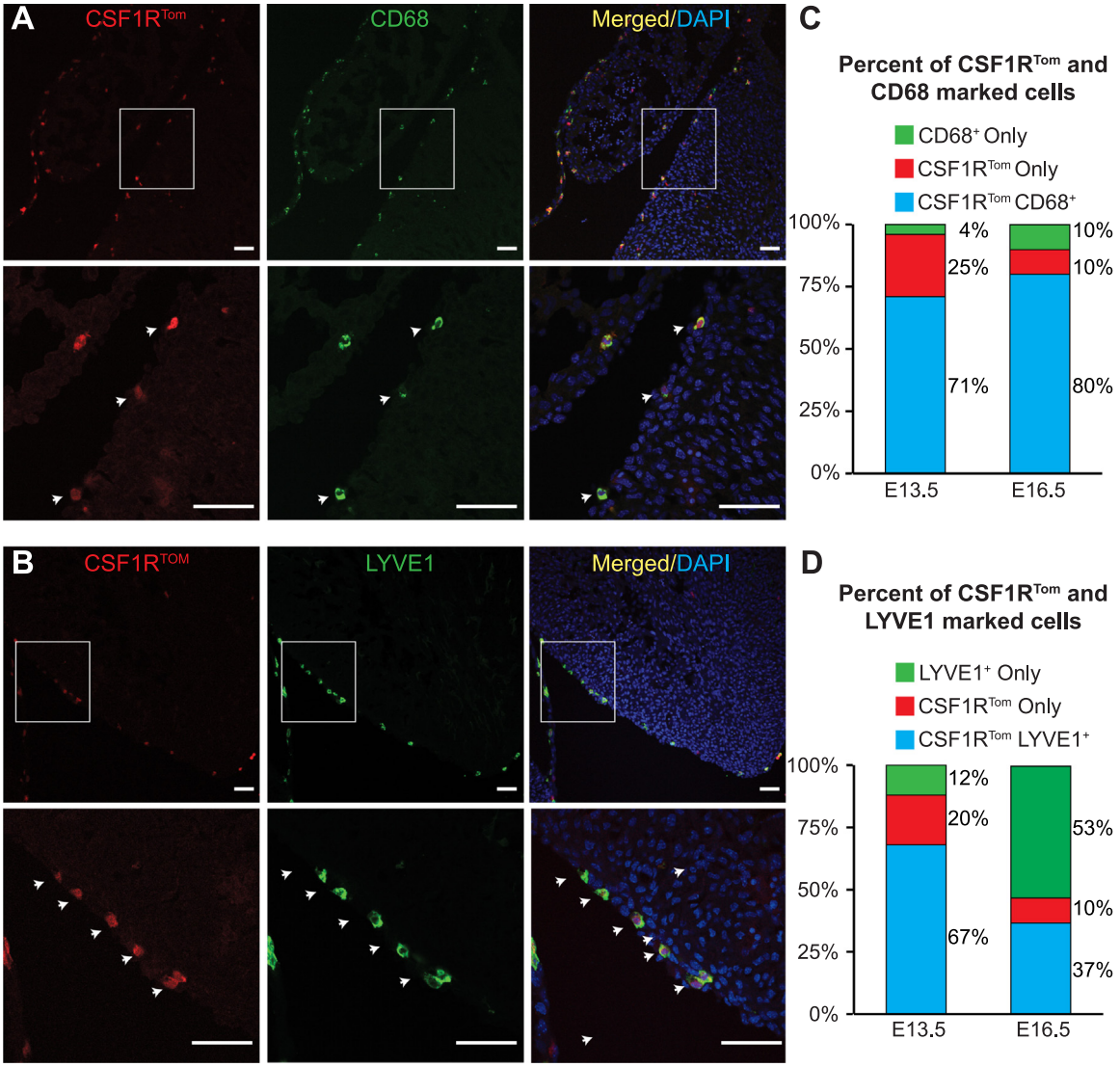

Fig. 2. Csf1r^{MCM}-marked yolk sac macrophage lineage in the fetal heart. (A–B) Csf1r^{MCM}; Rosa26^{Tomato} embryos were labeled by tamoxifen treatment at E8.0. At E13.5, heart cryosections were immunostained to determine the relationship of Csf1r^{MCM}-lineage cells, identified by Tomato expression, to macrophage markers CD68 (A) and LYVE1 (B). Csf1r^{MCM}-lineage cells were found in the epicardial region and co-expressed CD68 and LYVE1 (arrows). Bar=50 μm. (C–D). Quantitative analysis of overlap of Csf1r^{MCM}-lineage cells with CD68 (C) or LYVE1 (D) expression at E13.5 and E16.5. $n=3$ embryos per stage.

2.2. A majority of epicardial LYVE1$^+$ cells develop from yolk sac macrophages

The yolk sac macrophage lineage seeds many tissues in the developing fetus (Schulz et al., 2012) and contributes to resident macrophages in the adult heart, brain, and liver (Epelman et al., 2014b). Yolk sac macrophages can be genetically labeled by delivering tamoxifen at E8 to transgenic Csf1r^{MerCreMer} (Csf1r^{MCM}) embryos (Schulz et al., 2012; Epelman et al., 2014a; Gomez Perdiguero et al., 2015). This pulse activation of Cre indelibly turns on expression of a reporter gene selectively in yolk sac macrophages, allowing their subsequent fate to be tracked. Macrophages formed by later waves of HSC-dependent hematopoiesis are not labeled by this pulse, because they and their precursors do not express Csf1r^{MCM} within this time window (Schulz et al., 2012; Epelman et al., 2014b; Gomez Perdiguero et al., 2015). To investigate the contribution of yolk sac macrophages to cardiac macrophages in the fetal heart, we administered tamoxifen at E8.5 to Csf1r$^{MCM/+}$; Rosa26^{Tomato} embryos. We confirmed that this regimen did not label later definitive hematopoietic cells marked by CD41 (Robin et al., 2011) in the AGM or "hemogenic endocardium" of the developing heart (Nakano et al., 2013) (Suppl. Fig. 4). At E13.5, we analyzed co-expression of the genetic lineage tracer (Tomato) and the macrophage markers CD68 and LYVE1 (Fig. 2A and B). Tomato-expressing cells were localized in the epicardial region of the heart (Fig. 2A and B). Quantitative analysis showed that 95% (71%/75%) and 89% (80%/90%) of epicardial CD68$^+$ cells co-expressed the genetic lineage tracer at E13.5 and E16.5, respectively (Fig. 2C). Collectively our data indicate that fetal heart macrophages predominantly originate from the fetal yolk sac. Because fetal demise limited tamoxifen dose, Csf1r^{MCM} labeling was incomplete and it is possible that the small fraction of cells (5%) lacking the Cre label at E13.5 derive from the same yolk sac macrophage lineage. However, the increase in the CD68$^+$ Tomato$^-$ population from 5% to 11.1% between E13.5 and E16.5 is unlikely to be explained by inefficient labeling; rather, this increase in the unlabeled population suggests that additional embryonic macrophage sources, such as HSC-dependent hematopoiesis in the fetal liver, make minor contributions to cardiac macrophages that increase at late gestation.

We also examined the relationship of LYVE1 expression to fetal yolk sac macrophages in the heart. Consistent with the above CD68 analysis, at E13.5 the large majority (85%) of LYVE1$^+$ cells co-expressed the fetal yolk sac macrophage genetic lineage tracer (Fig. 2B and D). Because of the expansion of the LYVE1$^+$ cardiac lymphatics, by E16.5 most LYVE1$^+$ cells no longer co-expressed the fetal yolk sac macrophage genetic lineage tracer (Fig. 2D).

Together, our data indicate the fetal yolk sac macrophages seed the epicardial region of the fetal heart and largely co-express CD68 and LYVE1.

2.3. Epicardial ablation reduces epicardial macrophages

Because fetal heart macrophages are concentrated in the epicardial region, we hypothesized that the epicardium is required for their recruitment or retention. To test this hypothesis, we ablated the fetal epicardium by selective expression of diphtheria toxin (DTA) (Ivanova et al., 2005) in epicardial cells and asked if this

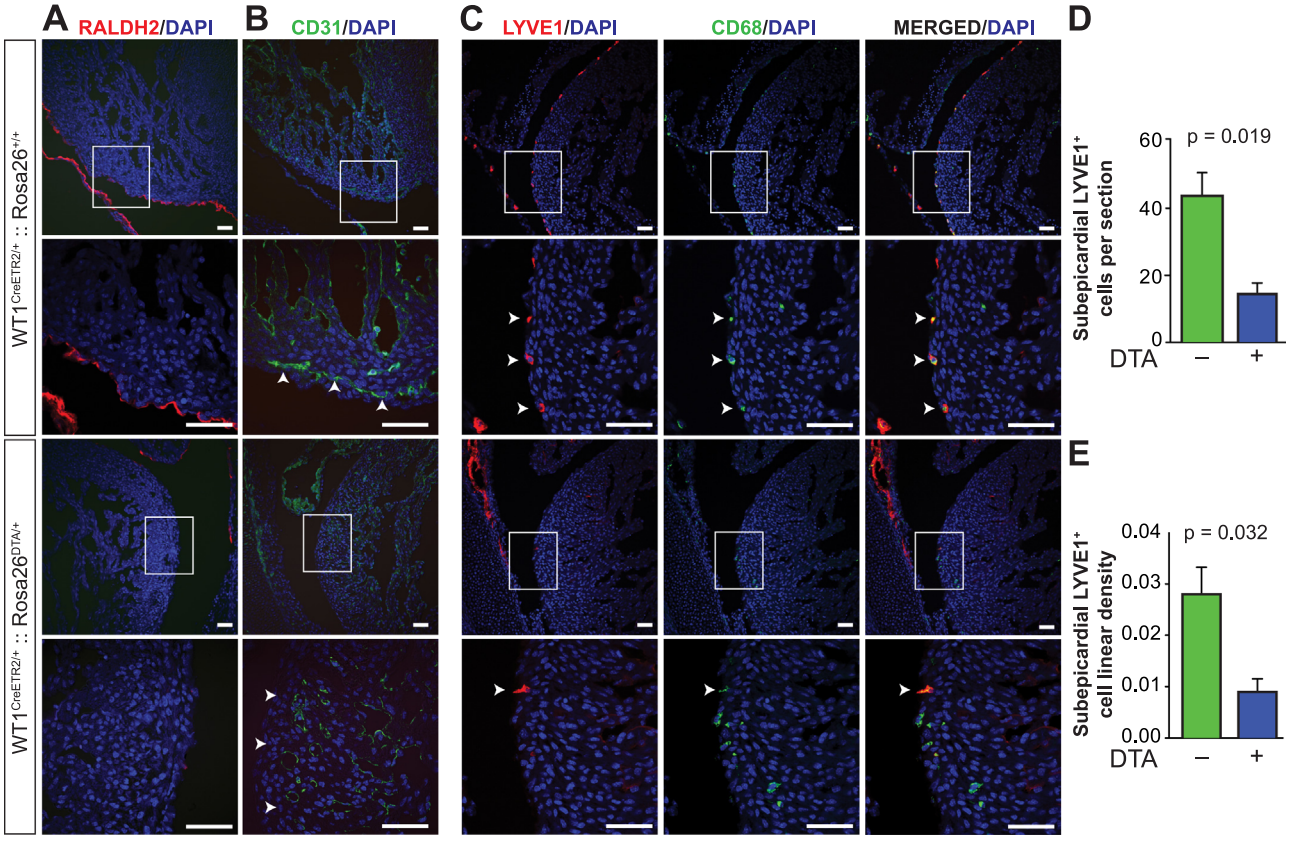

Fig. 3. Epicardial ablation caused defective macrophage population of the fetal heart. Epicardium was ablated by treatment of Wt1$^{CreERT2/+}$; Rosa26$^{DTA/+}$ embryos with tamoxifen at E11–E11.5. Hearts were analyzed at E14–E14.5. (A) Staining for epicardial marker RALDH2 confirmed efficient ablation of most of the ventricular epicardium. (B) CD31 staining showed disruption of coronary vessel development in the subepicardium. (C) Double staining with LYVE1 and CD68 demonstrated reduction of LYVE1$^+$ CD68$^+$ macrophage cells in the subepicardial region in hearts with ablated epicardium. (D) Quantitation of the number of subepicardial LYVE1$^+$ cells per heart section. (E) Quantitation of the linear density of subepicardial LYVE1$^+$ cells. The number of cells was divided by the length of the subepicardial region (measured in μm). Bars represent mean ± SEM. $n=4$. Groups were compared using Welch's t-test. White bar = 50 μm.

impeded cardiac macrophage recruitment. $Wt1^{CreERT2}$; $Rosa26^{DTA}$ mice were treated with one dose of tamoxifen at E11–11.5 to induce epicardial Cre activity, triggering epicardial DTA expression and thereby eliminating the expressing cells. Immunohistochemistry for epicardial marker RALDH2 confirmed successful epicardial ablation (Fig. 3A). Loss of epicardium function was confirmed by disruption of coronary vascular development (Fig. 3B), which requires signaling from epicardium (Tian et al., 2015). Loss of epicardium reduced the number of epicardial $LYVE1^+$ and $CD68^+$ cells (Fig. 3C). Quantitative analysis showed that in animals with epicardial ablation there was a significant reduction of the total number of $LYVE1^+$ epicardial macrophages compared to hearts with intact epicardium (Fig. 3D; $P=0.019$). This reduction was also evident if we adjusted for the circumference of the heart section being analyzed (Fig. 3E; $P=0.032$).

Together these data indicate that the epicardium promotes yolk sac macrophage recruitment or retention in the fetal heart.

2.4. *WT1 is required for accumulation of epicardial macrophages*

The transcription factor WT1 is expressed in the epicardium and is required for many of its hallmark characteristics (Moore et al., 1999; von Gise et al., 2011). Therefore we hypothesized that *Wt1* is required for epicardium to recruit or retain yolk sac macrophages in the epicardial region. To test this hypothesis we used the $WT1^{CreERT2}$ and $WT1^{GFPCre}$ knockin alleles, which express the CreERT2 or GFPCre fusion proteins, respectively, and do not express *Wt1* (Zhou et al., 2008; von Gise et al., 2011). $Wt1^{GFPCre/CreERT2}$ hearts exhibited abnormal coronary vessel development (Fig. 4A and B) as we previously reported (von Gise et al., 2011), confirming *Wt1* loss of function. $Wt1^{GFPCre/CreERT2}$ hearts showed loss of $LYVE1^+$ foci, indicating impaired macrophage recruitment from the yolk sac (Fig. 4C). When quantified, we found that the number of $LYVE1^+$ foci in whole mount preparations of $Wt1^{GFPCre/CreERT2}$ hearts were significantly reduced compared to the heterozygous and wild-type hearts (Fig. 4D).

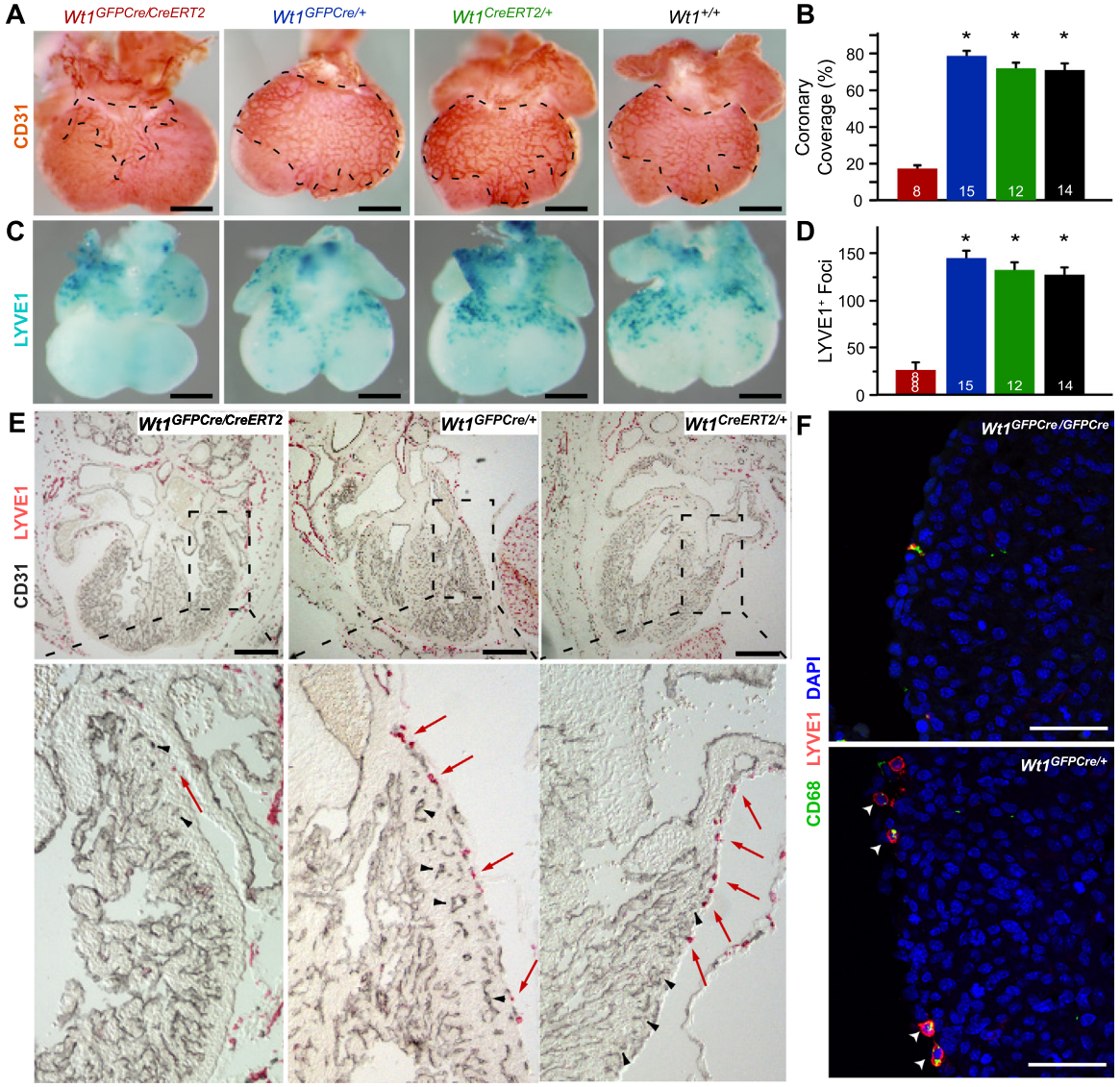

Fig. 4. Epicardial Wt1 inactivation impairs macrophage population of the fetal heart. $Wt1^{GFPCre/CreERT2}$ knockout embryos were compared to littermate controls at E13.5. (A–B) Whole-mount staining for endothelial cell marker CD31. Black dotted lines indicate the extent of the coronary vascular plexus overlying the myocardium. Quantitation of the fraction of the myocardium covered by the coronary vascular plexus demonstrated severe deficiency of coronary vascular development in *Wt1* loss of function. (C–D) Whole-mount staining for macrophage marker LYVE1. Over the ventricles, LYVE1 staining appeared as blue foci. Quantitation of the number of foci demonstrated a paucity of $LYVE1^+$ staining in *Wt1* loss of function. (E–F) Double immunohistochemical (E) or immunofluorescent (F) detection of CD31 and LYVE1 in heart sections. In E, $LYVE1^+$ macrophages are marked by red arrows, while $CD31^+$ coronary endothelial cells are indicated by black arrowheads. In F, white arrowheads indicate $CD68^+$ $LYVE1^+$ cells. *, $p<0.01$ using Welch's *t*-test. Bars represent mean ± SEM. Sample numbers are shown within bars.

These findings were confirmed on heart sections stained with LYVE1 or CD68, in which there was a paucity of LYVE1$^+$ or CD68$^+$ epicardial cells in hearts lacking WT1 (Fig. 4E). Together, these data show that *Wt1* promotes epicardial recruitment or retention of macrophages in the fetal heart.

3. Discussion

Recent work has shown that yolk sac macrophages give rise to a subset of adult heart resident macrophages that play important roles in promoting cardiac repair and regeneration (Epelman et al., 2014a; Lavine et al., 2014). Here we show that the epicardium is required to recruit yolk sac macrophages to the fetal heart during embryonic development. Our work shows that developmentally regulated, tissue-specific signaling mechanisms are responsible for embryonic macrophages to be recruited to their target organs.

Fetal heart macrophages are attracted to a niche located within or just below the epicardium, where they are closely related to the nascent coronary vasculature. Our study suggests that this niche promotes the selective recruitment or retention of Csf1r^{MCM}-lineage, CCR2$^-$ macrophages, compared to non-Csf1r^{MCM}, CCR2$^+$ macrophages. Prior work has established that the Csf1^{MCM} macrophage lineage originates from the fetal yolk sac, whereas non-Csf1r^{MCM} lineage, CCR2$^+$ macrophages originate from definitive hematopoiesis in the fetal liver and begin to emerge after E15.5 (Schulz et al., 2012; Epelman et al., 2014b; Gomez Perdiguero et al., 2015). Our data showing that CD41$^+$ hemogenic endothelium in the AGM were not labeled by Csf1^{MCM} is consistent with these prior observations and the origin of Csf1^{MCM} macrophages from the yolk sac. The epicardial niche occurs at the interface of the myocardium and epicardium and is characterized by a rich milieu of paracrine signals, nascent coronary vessels, distinctive extracellular matrix, and subepicardial mesenchymal cells derived from epicardium through EMT. Disruption of one or more of these features of the epicardial niche by epicardial ablation likely accounts for loss of yolk sac macrophage recruitment/retention.

Our work also identifies WT1 as a transcriptional regulator of the epicardial program required for fetal yolk sac macrophage recruitment to the heart. One potential mechanism by which WT1 regulates yolk sac macrophage recruitment is through the expression of cell adhesion or paracrine signaling molecules. Adult epicardial cells express the chemotactic factor monocyte chemoattractant protein-1 (Zhou et al., 2011), the ligand for CCR2, which is expressed by circulating monocytes. However, our data show that fetal LYVE1$^+$ CD68$^+$ macrophages are largely CCR2 negative. Fetal yolk sac macrophages expressed *Csf1r*, but epicardial expression of its ligand was not altered in *Wt1* loss of function (data not shown). Although the epicardium is present in *Wt1* null hearts, the subepicardial architecture is grossly abnormal (Moore et al., 1999; von Gise et al., 2011). Thus it is also possible that *Wt1* ablation impacts yolk sac macrophage recruitment indirectly, through loss of the normal architecture or cell types of the epicardial niche.

Our study indicates that organ-specific programs are required to recruit embryonic macrophages to their future abodes, and identifies the epicardium and the epicardial niche as critical for yolk sac macrophage recruitment to the heart. Given that most visceral organs including the liver are enveloped by mesothelial cells closely related to the epicardium, it is likely that analogous signals contribute to their recruitment of embryonic macrophages. It will also be interesting to determine the extent to which the epicardium and the epicardial niche contribute to the organ specific education of the recruited macrophages.

4. Experimental procedures

4.1. Mice

Studies were performed under protocols approved by the Boston Children's Hospital Institutional Animal Care and Use Committee. Wt1^{CreERT2}, Wt1^{GFPCre}, Csf1r^{MerCreMer}, ROSA26$^{eGFP\text{-}DTA}$, and ROSA26^{tdTomato} mouse lines have been described previously (Zhou et al., 2008; Qian et al., 2011; Ivanova et al., 2005; Madisen et al., 2010) and were obtained from Jackson Labs. For fetal labeling, tamoxifen was administered by gavage at a dose of 0.12 mg/g maternal body weight. Timed pregnant CFW mice were obtained from Taconic Laboratories.

4.2. Flow cytometry and cell sorting

For FACS analysis, whole CFW embryo hearts were collected between 13.5 and 14 embryonic days in cold PBS and enzymatically dissociated (Miltenyi Neonatal Heart Dissociation Kit). Red blood cells were removed using RBC lysis buffer (Miltenyi). Cells were blocked with 10% FBS before antibody staining. Antibodies are listed in Supplementary table 1. Staining was done one ice in PBS for 45 min with 0.2 μg/mL of the antibody per 10^6 cells. Cells were counted using a Countess automated cell counter (Invitrogen). After washing with cold PBS, samples were resuspended in 300 μl FACS Buffer (1 × PBS, Ca/Mg free, pH7.4, with 0.5% BSA, 1 mM EDTA and 0.05% NaN_3). Flow cytometry was performed on a BD FACSAria™ II instrument and analyzed using Becton Dickinson Diva software, version 6.11. For RNA isolation, sorted cells were preserved in either 750 μl of TRIzol reagent (Life Technologies) or 350 μl of RLT buffer (Qiagen) + β-mercaptoethanol.

4.3. Histology

Embryos were collected and washed in cold PBS, and fixed in 4% PFA for 4 h at 4 °C then washed for 1 h in PBS. For cryosections, fixed embryos were incubated in 30% sucrose overnight at 4 °C, equilibrated in OCT for 1–2 h at 4 °C and the frozen in 2-butylbutane cooled in dry ice. Cryopreserved tissues were cut with a cryostat in 8–12 μm thick sections, air dried briefly, and stored at −20 °C.

Tissue sections were washed and permeabilized in PBS+0.1% Triton-X100 (PBST) for 15 min and blocked with PBST+5% normal donkey serum (PBSST) for 1 h at room temperature. Tissue sections were incubated with primary antibodies (Supplementary table 1) overnight at 4 °C, washed with PBST 3 times 10 min, and then incubated with secondary antibody for 90 min at room temperature. All secondary antibodies were Alexa flurophore (488, 555, 647) conjugates developed in donkey (Life Technologies) and applied at dilutions of 1:250–1:500 in PBST. Sections were further washed in PBST, counter stained with DAPI (Life Technologies), and mounted in Vectashield (Vector). Sections were imaged on an FV1000 confocal microscope.

To quantitate of immunostaining data, for each group we counted all subepicardial cells in at least 2 non-sequential sections per embryo for at least 3 embryos.

4.4. RNA Preparation at RT-qPCR

RNA was isolated using TRIzol reagent (Life Technologies) or the Qiagen RNeasy mini kit with on-column DNase digestion. RNA was reverse transcribed using the SuperScript III First-Strand Synthesis SuperMix (Life Technologies) with random primers. qPCR was performed on a BioRad CFX96 real time PCR system with Sybr green qPCR master mix (Life Technologies). Primers (Supplementary table 2) were validated to yield efficient amplification

products and a single peak melting curve. Relative gene expression was calculated with the ΔΔCt method and normalized to *Gapdh*.

4.5. Statistics

Results are shown as mean ± SEM. Comparison between two groups was performed using Welch's *t*-test and $P < 0.05$ was defined as statistically significant.

Author contributions

SMS and AVG contributed equally to this study. SMS and AVG designed, performed, and analyzed the experiments. SMS wrote the manuscript with contributions from AVG. BZ analyzed *Wt1* null embryos for LYVE1 expression. WTP designed experiments, analyzed data, and wrote the manuscript.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2016.03.014.

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