JOURNAL OF THE AMERICAN COLLEGE OF CARDIOLOGY VOL. 73, NO. 23, 2019

Pro-Angiogenic Macrophage Phenotype to Promote Myocardial Repair

Bartolo Ferraro, PhD,[a,b,*] Giovanna Leoni, PhD,[a,b,*] Rabea Hinkel, DVM,[b,c,d] Steffen Ormanns, MD,[e] Nicole Paulin, DVM,[a,b] Almudena Ortega-Gomez, PhD,[a,b] Joana R. Viola, PhD,[a,b] Renske de Jong, PhD,[a] Dario Bongiovanni, MD,[b,c] Tarik Bozoglu, PhD,[c] Sanne L. Maas, MSc,[a,b] Michele D'Amico, PhD,[f] Thorsten Kessler, MD,[b,g] Tanja Zeller, PhD,[h,i] Michael Hristov, MD,[a] Chris Reutelingsperger, PhD,[j] Hendrik B. Sager, MD,[b,g] Yvonne Döring, PhD,[a,b] Matthias Nahrendorf, MD,[k] Christian Kupatt, MD,[b,c] Oliver Soehnlein, MD, PhD[a,b,l]

ABSTRACT

BACKGROUND Heart failure following myocardial infarction (MI) remains one of the major causes of death worldwide, and its treatment is a crucial challenge of cardiovascular medicine. An attractive therapeutic strategy is to stimulate endogenous mechanisms of myocardial regeneration.

OBJECTIVES This study evaluates the potential therapeutic treatment with annexin A1 (AnxA1) to induce cardiac repair after MI.

METHODS AnxA1 knockout (*AnxA1*$^{-/-}$) and wild-type mice underwent MI induced by ligation of the left anterior descending coronary artery. Cardiac functionality was assessed by longitudinal echocardiographic measurements. Histological, fluorescence-activated cell sorting, dot blot analysis, and in vitro/ex vivo studies were used to assess the myocardial neovascularization, macrophage content, and activity in response to AnxA1.

RESULTS *AnxA1*$^{-/-}$ mice showed a reduced cardiac functionality and an expansion of proinflammatory macrophages in the ischemic area. Cardiac macrophages from *AnxA1*$^{-/-}$ mice exhibited a dramatically reduced ability to release the proangiogenic mediator vascular endothelial growth factor (VEGF)-A. However, AnxA1 treatment enhanced VEGF-A release from cardiac macrophages, and its delivery in vivo markedly improved cardiac performance. The positive effect of AnxA1 treatment on cardiac performance was abolished in wild-type mice transplanted with bone marrow derived from *Cx$_3$cr1cre^{ERT2}Vegf$^{flox/flox}$* or in mice depleted of macrophages. Similarly, cardioprotective effects of AnxA1 were obtained in pigs in which full-length AnxA1 was overexpressed by use of a cardiotropic adeno-associated virus.

CONCLUSIONS AnxA1 has a direct action on cardiac macrophage polarization toward a pro-angiogenic, reparative phenotype. AnxA1 stimulated cardiac macrophages to release high amounts of VEGF-A, thus inducing neovascularization and cardiac repair. (J Am Coll Cardiol 2019;73:2990-3002) © 2019 The Authors. Published by Elsevier on behalf of the American College of Cardiology Foundation. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

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From the [a]Institute for Cardiovascular Prevention (IPEK), Ludwig-Maximilians-Universität Munich, Munich, Germany; [b]Deutsches Zentrum für Herz-Kreislaufforschung (DZHK), partner site Munich Heart Alliance, Munich, Germany; [c]Medizinische Klinik I, TU Munich, Germany; [d]Deutsches Primatenzentrum GmbH, Leibniz-Institut für Primatenforschung, Department of Laboratory Animal Science, Göttingen, Germany; [e]Institute of Pathology, Faculty of Medicine, LMU Munich, Munich, Germany; [f]Department of Experimental Medicine, University of Campania, Campania, Italy; [g]Department of Cardiology, German Heart Center Munich, Munich, Germany; [h]DZHK, Partner Site Hamburg/Kiel/Lübeck, Hamburg, Germany; [i]Clinic for Cardiology, University Heart Center, Hamburg, Germany; [j]Department of Biochemistry, Cardiovascular Research Institute Maastricht, University Maastricht, Maastricht, the Netherlands; [k]Center for Systems Biology, Massachusetts General Hospital, Harvard Medical School, Boston, Massachusetts; and the [l]Department of Physiology and Pharmacology (FyFa) and Department of Medicine, Karolinska Institutet, Stockholm, Sweden. *Drs. Ferraro and Leoni contributed equally to this work. The authors' research is supported by the Deutsche Forschungsgemeinschaft (SFB914 TP B08, SFB1123 TP A06 and B05, SO876/6-1, SO876/11-1, SA 1668/5-1), the German Centre for Cardiovascular Research, the Fritz Thyssen foundation, the Vetenskapsrådet (2017-01762), Deutsche Herzstiftung (F/28/17), the Else-Kröner-Fresenius Stiftung, European Research Council (STRATO 759272), the European Union's Horizon 2020 research and innovation program under the Marie Skłodowska-Curie grant agreement No 675111, and the People Program (Marie Curie Actions) of the European Union's Seventh Framework Program (FP7/2007-2013) under REA grant agreement n° 608765. Dr. Nahrendorf has

ISSN 0735-1097 https://doi.org/10.1016/j.jacc.2019.03.503

Coronary artery disease resulting in myocardial infarction (MI) is a major cause of death worldwide (1). Despite the advent of new therapeutic strategies to restore blood flow, we are not yet able to prevent the onset of heart failure following MI. Hence, it is a major challenge to identify innovative strategies to restore nutrient supply to the infarcted myocardium, ultimately aimed at regeneration of myocardial functionality.

The cellular response following MI is characterized by a rapid recruitment of neutrophils. Their arrival is superseded by the infiltration of classical monocytes, which contribute to clearance of debris (2). However, this subset also drives robust inflammation, leading to pathological remodeling. In contrast, the appearance of nonclassical monocytes and reparative macrophages marks a turning point between inflammation and its resolution, as these cells govern repair and angiogenesis. At this point, knowledge about mechanisms regulating this cellular switch and about origin and identity of molecular cues involved is scarce.

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Annexin A1 (AnxA1) is quickly released upon cellular stress (3); it acts through Formyl peptide receptor-2 to prevent chemokine-mediated integrin activation, and thus, turns off inflammatory recruitment of myeloid cells (4). AnxA1 also activates pro-repair mechanisms by activation of Rac1 and NOX1, resulting in enhanced epithelial cell migration after injury (5). Local intestinal delivery of an AnxA1 fragment encapsulated within polymeric nanoparticles accelerated recovery following experimentally induced colitis (6,7). With its central position during the switch from inflammation to resolution, we hypothesized that AnxA1 may be an important cue linking initial myeloid cell recruitment to myocardial repair. Using $AnxA1^{-/-}$ mice and therapeutic delivery of AnxA1, we demonstrate that myeloid cell-derived AnxA1 controls the production of pro-angiogenic vascular endothelial growth factor (VEGF)-A from reparative macrophages, an effect also reproduced in a large animal model of MI.

METHODS

MICE. Wild-type (WT) female C57BL/6 mice were purchased from Janvier (Le Genest-Saint-Isle, France). $AnxA1^{-/-}$ mice were a gift from Roderick Flower (Barts and The London School of Medicine and Dentistry, London, United Kingdom). Mice were housed under a standard day/night cycle, with free access to food and water. All animal experiments were approved by the local ethics committee.

MOUSE STUDIES. Mice were anaesthetized, ventilated, and a left-sided thoracotomy was performed. The left anterior descending coronary artery (LAD) was ligated with 1 single suture. Post-operative analgesia was given subcutaneously upon induction of myocardial infarction, as well as 8 and 24 h post-surgery. In treatment studies, human recombinant AnxA1 (hrAnxA1) was administered (10 μg, intraperitoneally [i.p.]) daily up to 6 days post-MI. This dosing schedule was chosen based on published data (6).

For macrophage depletion, clodronate-filled liposomes were administered (100 μl, i.p.) 24 h before surgery as well as every other day after MI. For bone marrow transplantation, donor bone marrow cells ($\sim 3 \times 10^6$) from $Cx_3cr1cre^{ERT2}Vegf^{flox/flox}$ were administered to WT mice 1 day after whole body irradiation. At 4 weeks after transplantation, mice were treated with tamoxifen (1 mg dissolved in 50 μl Miglyol [Sasol, Witten, Germany], 20 g of weight) to induce macrophage VEGF depletion.

Transthoracic echocardiography was performed with a Vevo3100 Imaging system equipped with a 40MHz transducer (VisualSonics, Amsterdam, the Netherlands). Left parasternal long-axis view and left midpapillary, apical, and basal short-axis views were acquired.

RESULTS

ANNEXIN A1 PRESERVES CARDIAC FUNCTIONALITY. Following MI, circulating neutrophils and monocytes migrate into the infarcted myocardium and govern the ensuing inflammatory and reparative processes (8). Timely resolution of inflammation requires the coordinated actions of several different proresolving mediators, including AnxA1. In our experiments, AnxA1 accumulation in the infarcted heart peaked at day 2 after MI and subsequently declined (**Figure 1A**). Circulating myeloid cells are the main AnxA1 sources (**Figure 1B**), suggesting that these may deliver AnxA1 to the infarcted myocardium. In fact, AnxA1 in

ABBREVIATIONS AND ACRONYMS

AnxA1 = annexin A1

FGF-b = basic fibroblast growth factor

LAD = left anterior descending coronary artery

MI = myocardial infarction

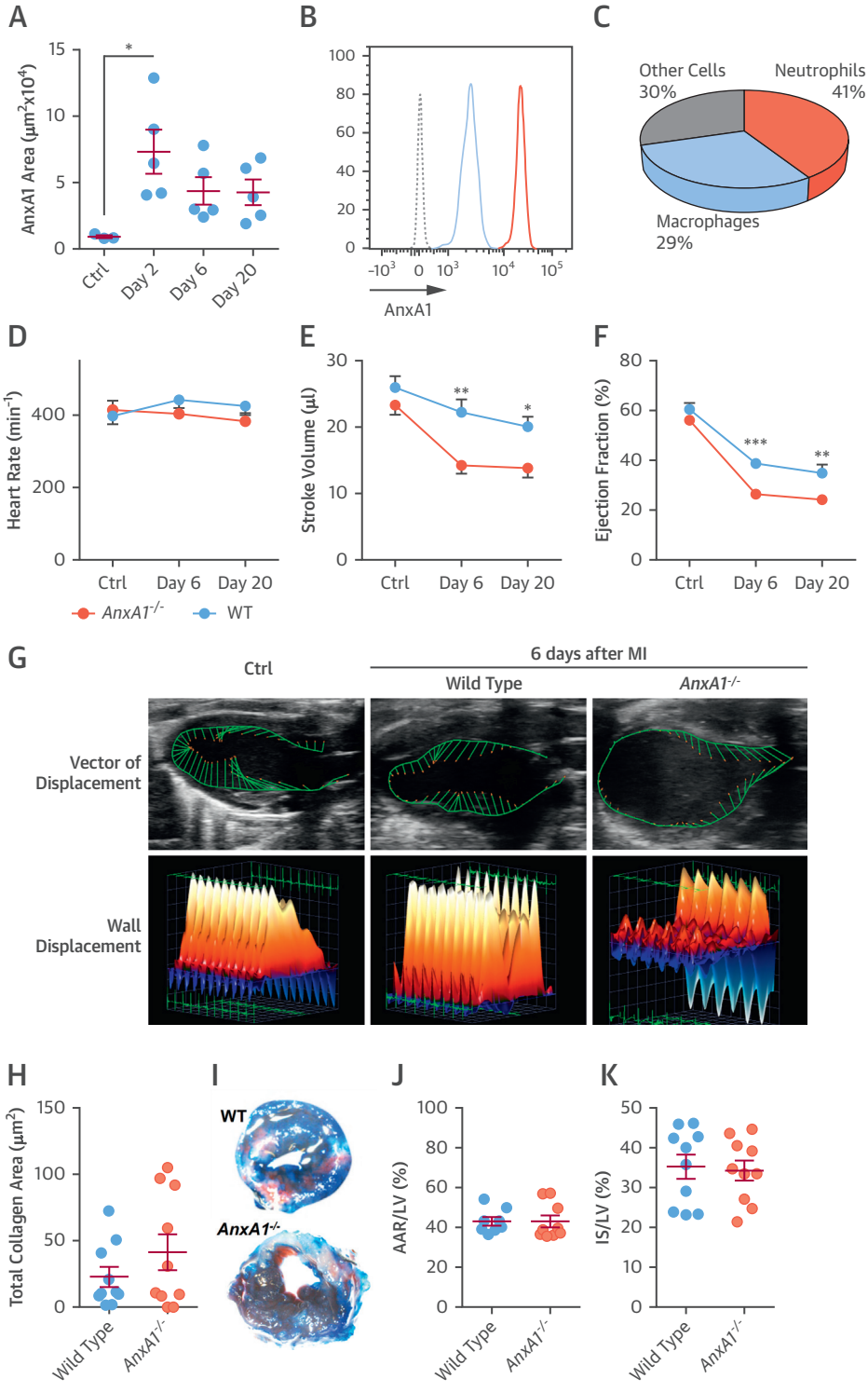
VEGF-A = vascular endothelial growth factor A

WT = wild type

served as a consultant for Verseau, IFM Therapeutics, and Sigilon; and has received research support from Behring, Medtronic, Novartis, and GlaxoSmithKline. All other authors have reported that they have no relationships relevant to the contents of this paper to disclose.

Manuscript received November 4, 2018; revised manuscript received March 12, 2019, accepted March 19, 2019.

FIGURE 1 Annexin A1 Promotes Cardiac Repair After Myocardial Infarct



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infarcted hearts primarily derives from infiltrating neutrophils (Figure 1C).

To investigate the role of endogenous AnxA1 in myocardial recovery, longitudinal echocardiographic measurements in WT and *AnxA1*$^{-/-}$ mice were performed. These analyses demonstrated that *AnxA1*$^{-/-}$ mice displayed a worsened cardiac performance compared with the WT group. In particular, a lack of AnxA1 led to a reduction of the stroke volume and ejection fraction (Figures 1D to 1F). This reduced global performance was also reflected by mitigated left ventricular contractility (Figure 1G).

To study mechanisms underlying reduced cardiac performance, we evaluated the fibrotic response in the ischemic myocardium. However, no differences in the cardiac collagen content between the groups were found (Figure 1H). In addition, infarct size was not different between the strains, a finding corroborated by lack of differences in plasma cardiac troponin I levels (Figures 1I to 1K, Online Figure 1A). Of note, a recent study reports that lack of AnxA1 increases infarct sizes upon ischemia-reperfusion (9), suggesting model-specific effects. Finally, the number of apoptotic cells did not differ (Online Figure 1B). Taken together, a lack of AnxA1 reduces cardiac recovery post-MI, which cannot be attributed to fibrosis, infarct extent, or accumulation of dead cells.

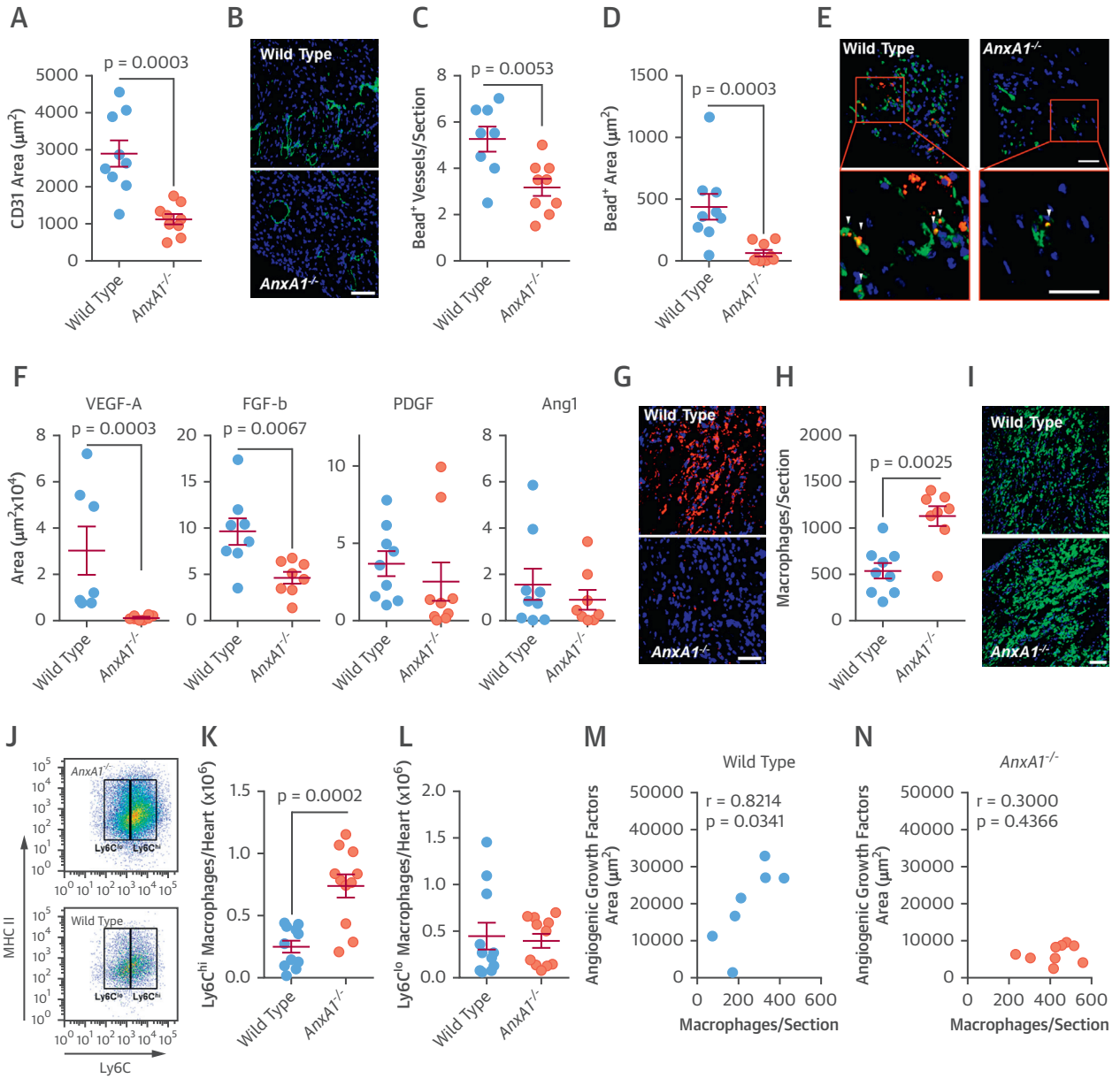
ANNEXIN A1 PROMOTES MYOCARDIAL ANGIOGENESIS AND LIMITS ACCUMULATION OF INFLAMMATORY MACROPHAGES. The post-MI phase is characterized by a reparative stage where angiogenesis occurs. At 6 days, post-MI microvascular density was significantly reduced in *AnxA1*$^{-/-}$ mice compared with WT mice (Figures 2A and 2B), suggesting hampered angiogenesis in mice lacking AnxA1. This notion was confirmed following intravenous infusion of fluorescent microbeads allowing visualizing functional microvasculature (Figures 2C to 2E), as well as by quantification of newly formed CD105^{+} endothelial cells (Online Figure 1C). Angiogenic growth within damaged tissues provides nutrient supply to facilitate repair. These events are governed by several growth factors, including VEGF-A, basic fibroblast growth factor (FGF-b), platelet-derived growth factor, and Ang1. Here, we compared the myocardial expression of these angiogenic factors in WT and *AnxA1*$^{-/-}$ mice (Figures 2F and 2G). VEGF-A was largely depleted in the absence of AnxA1. Expression of pro-angiogenic FGF-b was also reduced in mice lacking AnxA1, whereas no differences were observed for platelet-derived growth factor and Ang1.

At the injury site, macrophages remove necrotic debris, secrete cytokines and growth factors, and modulate the angiogenic response. Consequently, we analyzed the accumulation of macrophages in the myocardium post-MI. In absence of AnxA1, cardiac macrophage content was significantly higher (Figures 2H and 2I), a matter likely attributable to the antiadhesive effects of AnxA1 (4). Of note, the number of blood leukocyte subsets was not different between the mouse strains (Online Figure 2). Given the plasticity of macrophages, we examined not just their numbers, but also their polarization. In contrast to WT mice, cardiac macrophages from *AnxA1*$^{-/-}$ mice showed a clear antireparative, proinflammatory (Ly6C^{hi}) phenotype compared with WT mice (Figures 2J to 2L). The increase in macrophage numbers and a shift toward a proinflammatory phenotype is consistent with a previous report studying the effect of AnxA1 in ischemia-reperfusion (9). We next evaluated a possible connection between the 2 striking observations we made in *AnxA1*$^{-/-}$ mice, i.e., hampered angiogenesis and the overt accumulation of inflammatory macrophages. Thus, we correlated the numbers of macrophages and angiogenic growth factors in WT and *AnxA1*$^{-/-}$ mice. While we found a positive correlation in WT mice, this correlation was lost in mice lacking AnxA1 (Figures 2M and 2N). Taken together, the reparative response differed markedly in *AnxA1*$^{-/-}$ mice, indicating a

FIGURE 1 Continued

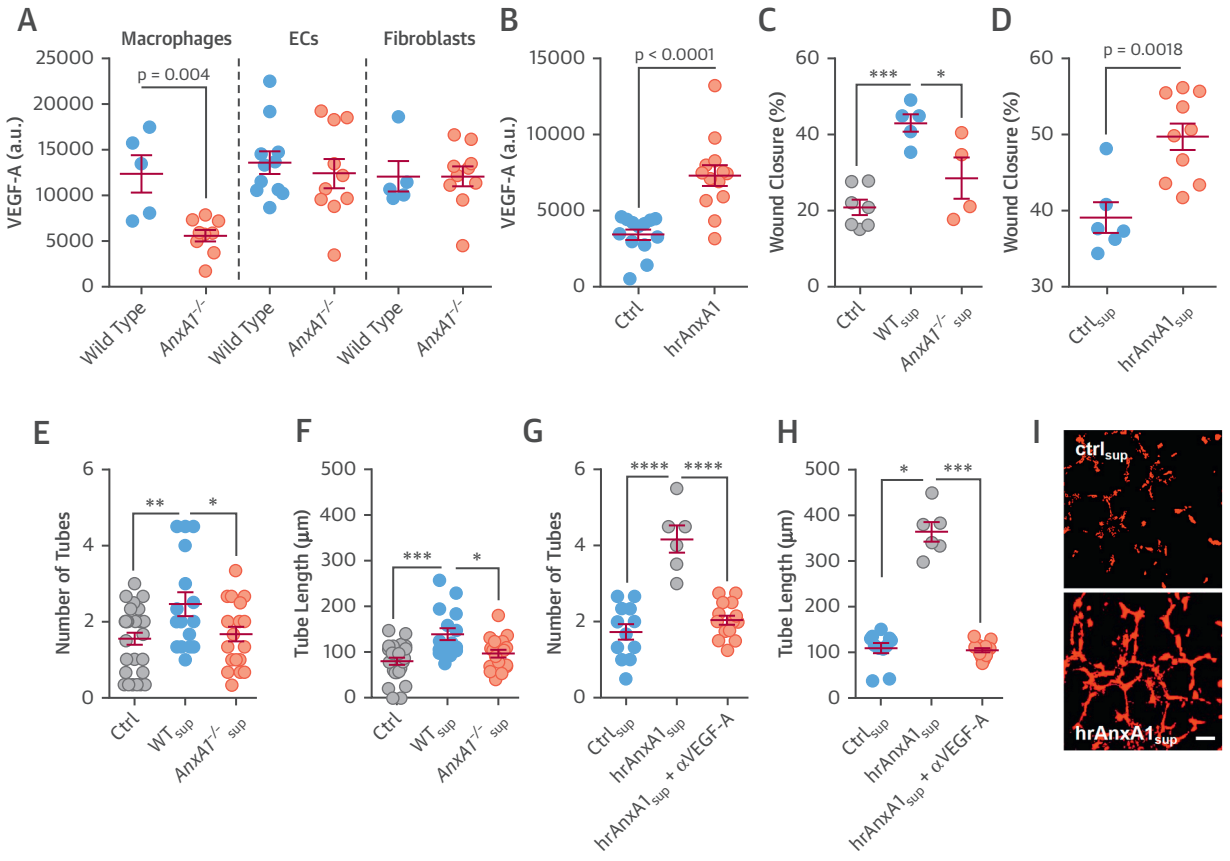
(A) Quantification of myocardial AnxA1 deposition at indicated days post-MI. One-way analysis of variance, *$p < 0.05$ in Dunnett's multiple comparison test. **(B)** Representative histogram of intracellular AnxA1 content in circulating neutrophils **(red)** and Ly6C^{high} monocytes **(blue)** compared with FMO control **(dotted)**. **(C)** Cellular distribution of myocardial AnxA1 expression in neutrophils, macrophages, and other cell types (pericytes, myofibroblasts, and cardiomyocytes) 2 days after MI. n = 4. **(D to K)** MI was induced in WT and *AnxA1*$^{-/-}$ mice. **(D)** Heart rate, **(E)** stroke volume, and **(F)** ejection fraction were assessed longitudinally by echocardiography. n = 7, 2-way analysis of variance with Bonferroni post hoc evaluation, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ between WT and *AnxA1*$^{-/-}$. **(G)** Vector diagrams showing the direction and magnitude of myocardial contraction at midsystole **(top)**. Three-dimensional regional wall displacement illustrations show contraction **(yellow-red)**, or relaxation **(blue)** of consecutive cardiac cycles. **(H to K)** Quantification of **(H)** total collagen, **(J)** area at risk, and **(K)** infarct size. **(I)** Representative images after Evans Blue injection and subsequent TTC staining. Values are mean ± SEM. Each **dot** in **A, H, J, and K** represents 1 mouse. Unpaired Student's *t*-test. AAR = area at risk; AnxA1 = Annexin A1; IS = infarct size; LV = left ventricle; MI = myocardial infarction; TTC = triphenyltetrazolium chloride; WT = wild type.

FIGURE 2 Annexin A1 Promotes Myocardial Angiogenesis and Limits Accumulation of Inflammatory Macrophages



(A to E) Quantification of microvascular density in infarcted hearts of WT and *AnxA1*$^{-/-}$ mice 6 days post-MI. **(A)** Quantification of CD31 staining and **(B)** representative images, unpaired Student's *t*-test. Scale bar, 100 μm. **(C to E)** Enumeration of microbeads injected intravenously and found **(C)** inside vessels and **(D)** per area, unpaired Student's *t*-test in **(C)** and Mann-Whitney *U* test in **(D)**. **(E)** Representative images of microbeads **(red)**, endothelium **(green)**, and DAPI **(blue)**. Scale bar, 100 μm. **(F)** Immunofluorescence-based quantification of indicated pro-angiogenic factors assessed in the infarct area of WT and *AnxA1*$^{-/-}$ mice, Mann-Whitney *U* test (VEGF), or unpaired Student's *t*-test (FGF-b). **(G)** Representative images of VEGF-A staining **(red)** and DAPI **(blue).** Scale bar, 50 μm. **(H)** Quantification and **(I)** representative images of macrophage accumulation in infarcted hearts of WT and *AnxA1*$^{-/-}$ mice. Mann-Whitney *U* test. Scale bar, 50 μm. **(J to L)** Fluorescence-activated cell sorting–based quantification of macrophage populations in the infarcted heart of WT and *AnxA1*$^{-/-}$ mice. **(J)** Representative blots displaying Ly6C^{high} and Ly6C^{low} macrophages as well as **(K and L)** their quantification, **(K)** unpaired Student's *t*-test, and **(L)** Mann-Whitney *U* test. **(M and N)** Pearson correlations between the number of cardiac macrophages and pro-angiogenic factors quantified in ischemic myocardium of **(M)** WT and **(N)** *AnxA1*$^{-/-}$ mice. Values are mean ± SEM. Each **dot** represents 1 mouse. Ang1 = angiopoietin 1; FGF-b = fibroblast growth factor b; MHC = major histocompatibility complex; PDGF = platelet-derived growth factor; VEGF-A = vascular endothelial growth factor; other abbreviations as in Figure 1.

FIGURE 3 Annexin A1 Stimulates Pro-Angiogenic VEGF-A Release From Macrophages

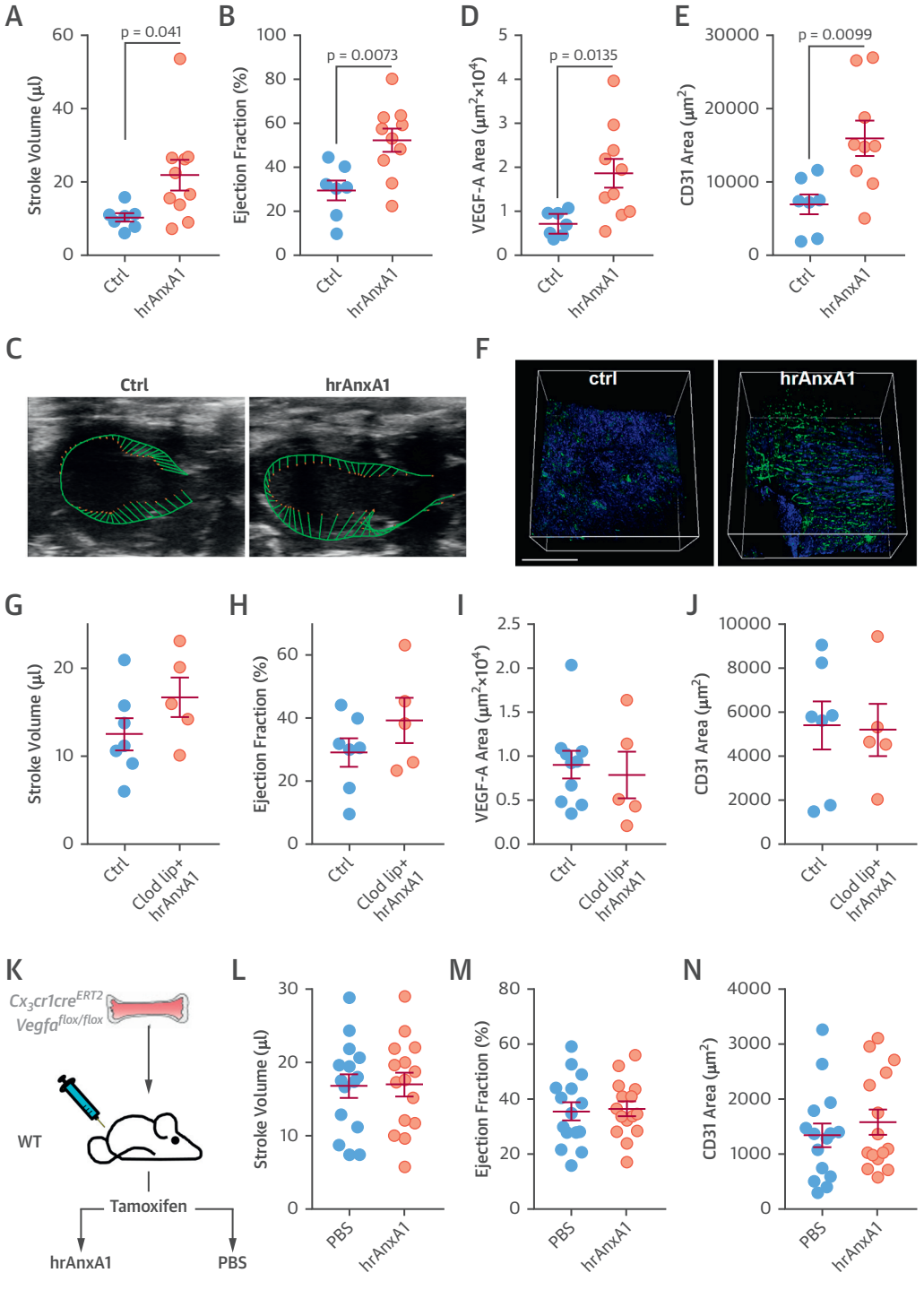


(A) Assessment of VEGF-A released in medium derived from cardiac macrophages, endothelial cells (EC) and fibroblasts that were sorted using fluorescence-activated cell sorting from WT or *AnxA1*$^{-/-}$ mice hearts 6 days post-MI, Mann-Whitney *U* test. **(B)** Quantification of VEGF-A released from WT cardiac macrophages isolated 6 days post-MI treated with phosphate-buffered saline (PBS) (ctrl) or AnxA1 (hrAnxA1, 100 nmol/l), Mann-Whitney *U* test. **(C)** Wound closure assay of endothelial cell monolayers in the presence of medium alone (ctrl), conditioned medium derived from cardiac WT macrophages (WT_{sup}) or in presence of conditioned media derived from *AnxA1*$^{-/-}$ cardiac ischemic macrophages (*AnxA1*$^{-/-}{}_{sup}$) 6 days post-MI, 1-way analysis of variance and Dunnett's multiple comparison test. **(D)** Wound closure assay of endothelial cell monolayers in the presence of conditioned medium derived from cardiac WT macrophages treated with PBS ($ctrl_{sup}$) or AnxA1 ($hrAnxA1_{sup}$), unpaired Student's *t*-test. **(E to I)** Endothelial cell tube formation assay. Endothelial cells were grown in basement membrane matrix and **(E and G)** tube number and **(F and H)** length were evaluated after treatment with medium alone (ctrl), conditioned medium derived from cardiac WT macrophages (WT_{sup}) or in presence of conditioned media derived from *AnxA1*$^{-/-}$ macrophages (*AnxA1*$^{-/-}{}_{sup}$) 6 days post-MI **E and F**. In separate experiments endothelial cells were treated with conditioned medium derived from cardiac WT macrophages treated with PBS ($ctrl_{sup}$), AnxA1 ($hrAnxA1_{sup}$), or AnxA1 in presence of a VEGF antibody ($hrAnxA1_{sup}$ + αVEGF-A). One-way ANOVA with Tukey multiple comparison test was used in **E, F, and G,** and Kruskal-Wallis test with Dunn's multiple comparison test was applied in **H**. **(I)** Representative images showing the effect of supernatant derived from hrAnxA1 stimulated macrophages treatment, compared with the control group. Endothelial cells were stained with CellMask Orange (Thermo Fisher, Waltham, Massachusetts). Scale bar, 100 μm. In **A**, each **dot** represents 1 mouse; **B to H**, each **dot** represents 1 well, in each well several fields of view were observed and averaged. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 between indicated groups. hrAnxA1 = human recombinant Annexin A1; other abbreviations as in **Figures 1 and 2**.

prominent role of AnxA1 on cardiac macrophages during the process of angiogenesis.

ANNEXIN A1 PROMOTES VEGF-A RELEASE FROM MACROPHAGES INDUCING ANGIOGENESIS. To identify the cellular origin of VEGF-A and FGF-b, both reduced in *AnxA1*$^{-/-}$ mice, we used fluorescence-activated cell sorting to sort cardiac macrophages, endothelial cells, and fibroblasts 6 days post-MI (Online Figure 3). The absence of AnxA1 led to a defect in VEGF-A secretion from cardiac macrophages (**Figure 3A**) as well as FGF-b release from cardiac fibroblasts (Online Figure 4). To test whether AnxA1 stimulated secretion of angiogenic growth factors from cardiac macrophages, we isolated cardiac macrophages post-MI from WT mice 6 days after MI and exposed these to AnxA1. Treatment in this way significantly enhanced the release of VEGF-A from cardiac

FIGURE 4 **Annexin A1 Delivery Improves Cardiac Functionality *Via* Release of VEGF-A From Macrophages**



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macrophages (**Figure 3B**, Online Figure 5). To assess the paracrine effects of the AnxA1-triggered release of angiogenic factors, we next treated murine endothelial cells with supernatant derived from cardiac macrophages harvested from WT or *AnxA1*$^{-/-}$ mice. Supernatant obtained from *AnxA1*$^{-/-}$ mice exhibited reduced endothelial cell activation as assessed in migration assays as well as in 3-dimensional tube formation assays (**Figures 3C, 3E, and 3F**). In addition, antibody-assisted neutralization of VEGF-A in supernatants of macrophages harvested from WT mice reduced angiogenic properties, an effect not observed when the antibody was used in supernatants of macrophages retrieved from *AnxA1*$^{-/-}$ mice (Online Figures 6A and 6B). The importance of VEGF-A is further supported by increased phosphorylation of VEGFR2 and the downstream signaling molecules focal adhesion kinase and AKT in endothelial cells treated with the supernatant of WT mice compared with endothelial cells incubated with supernatants of *AnxA1*$^{-/-}$ mice (Online Figures 6C to 6E). Additionally, endothelial cell death was significantly higher when the monolayer was exposed to the supernatant derived from cardiac *AnxA1*$^{-/-}$ macrophages compared with the supernatant derived from cardiac WT macrophages (Online Figure 7A). The opposite effect was found for endothelial cell proliferation (Online Figure 7B). Furthermore, supernatants of cardiac post-MI macrophages treated ex vivo with human recombinant AnxA1 (hrAnxA1) increased the angiogenic properties of endothelial cells (**Figures 3D and 3G to 3I**). In this setting, antibody-assisted neutralization of VEGF-A in supernatants of AnxA1-treated macrophages abrogated heightened angiogenic properties (**Figures 3G and 3H**). Together, these findings identify an indirect role of AnxA1 during neoangiogenesis through induction of VEGF-A release from cardiac macrophages.

ANNEXIN A1 IMPROVES MYOCARDIAL REPAIR THROUGH STIMULATING VEGF-A RELEASE FROM MACROPHAGES. To determine whether hrAnxA1-treatment could improve cardiac function, we administered hrAnxA1 to the mice daily, starting with the day of MI induction. In contrast to mice receiving vehicle, hrAnxA1 induced a significant improvement in cardiac function, evidenced by increased stroke volume, ejection fraction, and contractility (**Figures 4A to 4C**, Online Videos 1 and 2). In addition, hrAnxA1-treatment reduced infarct size while not affecting the dimensions of the area at risk (Online Figure 8). Consistent with our in vitro data, hrAnxA1 significantly increased myocardial VEGF-A expression as well as capillary density (**Figures 4D to 4F**). Furthermore, hrAnxA1 clearly increased the number of newly formed CD105^{+} endothelial cells (Online Figures 9A to 9D), thus confirming the angiogenic properties of AnxA1. Isolation of cardiac macrophages, endothelial cells, and fibroblasts from mice treated with hrAnxA1 demonstrated that only cardiac macrophages released higher amounts of VEGF-A under these conditions (Online Figure 10A). Furthermore, the fraction of cardiac ischemic macrophages expressing VEGF-A was significantly higher in mice treated with hrAnxA1 (Online Figure 10C). In contrast, FGF-b release from cardiac cells and FGF-b expression by macrophages in the ischemic myocardium was not altered in mice receiving hrAnxA1 (Online Figures 10B to 10D), suggesting that VEGF-A may stand out as a dominant angiogenic factor in the reparative responses evoked by hrAnxA1.

AnxA1 has previously been reported to exert its anti-inflammatory, reparative functions via either formyl-peptide receptor 1 (FPR1) or FPR2 (4,5). To test the involvement of these receptors in the AnxA1-mediated myocardial repair observed here, we administered hrAnxA1 to *Fpr1*$^{-/-}$ or *Fpr2*$^{-/-}$ mice after LAD ligation. In these experiments, hrAnxA1

FIGURE 4 Continued

(A to F) WT mice were treated with PBS or hrAnxA1 (10 μg/day/mouse, i.p.), and cardiac function and angiogenesis were assessed. **(A)** Stroke volume, unpaired Student's *t*-test. **(B)** Ejection fraction, unpaired Student's *t*-test. **(C)** Vector diagrams showing the direction and magnitude of myocardial contraction at midsystole (Online Videos 1 and 2). **(D)** Quantification of myocardial VEGF-A expression, unpaired Student's *t*-test. **(E)** Assessment of myocardial CD31 immunostaining, unpaired Student's *t*-test. **(F)** 3D reconstruction of CD31 staining acquired by 2-photon microscopy, scale bar, 200 μm. **(G to J)** Delivery of hrAnxA1 in mice depleted of macrophages fails to improve cardiac function. Clodronate liposomes were administered i.p. 24 h before the surgical procedure and on days 2, 4, and 6. **(G)** Stroke volume, unpaired Student's *t*-test. **(H)** Ejection fraction, unpaired Student's *t*-test. **(I)** Quantification of myocardial VEGF-A, unpaired Student's *t*-test. **(J)** Assessment of CD31 staining, unpaired Student's *t*-test. **(K to N)** Lack of VEGF-A in macrophages abrogates AnxA1-mediated myocardial recovery. **(K)** Experimental outline. WT mice were transplanted with bone marrow from *Cx*$_3$*cr1cre*^{ERT2}*Vegf*$^{flox/flox}$ mice and then treated with either hrAnxA1 or PBS post-MI. Evaluations of **(L)** stroke volume, **(M)** ejection fraction, and **(N)** neovascularization were performed 6 days post-MI, unpaired Student's *t*-test. All values are mean ± SEM. Each **dot** represents 1 mouse. hrAnxA1 = human recombinant Annexin A1; clod lip = clodronate liposomes; VEGF-A = vascular endothelial growth factor A; abbreviations as in **Figures 1 and 3**.

improved cardiac function in $Fpr1^{-/-}$ but not in $Fpr2^{-/-}$ mice (Online Figures 11A to 11B). In addition, hrAnxA1 promoted VEGF-A production and angiogenesis in mice lacking FPR1 but not in FPR2-deficient mice (Online Figures 11C to 11E). Thus, these data suggest that hrAnxA1 acts through FPR2 to promote cardiac repair.

To test if cardiac macrophages are effector cells during the reparative events triggered by AnxA1, we depleted cardiac macrophages using clodronate-filled liposomes. Interestingly, hrAnxA1 delivery in mice depleted of cardiac macrophages failed to improve cardiac function (**Figures 4G and 4H**). In line herewith, macrophage depletion abrogated increases in cardiac VEGF-A generation (**Figure 4I**) and neoangiogenesis (**Figure 4J**, Online Figure 9B). These results clearly indicate that macrophages are indispensable for hrAnxA1 to improve cardiac functionality.

To consolidate our observations, we transplanted WT mice with bone marrow from $Cx_3cr1cre^{ERT2}Vegf^{flox/flox}$ mice, the latter lacking the ability to release VEGF from macrophages. Following reconstitution and tamoxifen treatment, the LAD was ligated and mice were either treated with hrAnxA1 or phosphate-buffered saline (**Figure 4K**). In this setup, hrAnxA1 failed to improve stroke volume, ejection fraction, and angiogenesis (**Figures 4L to 4N**, Online Figure 9C). Taken together, these data suggest that hrAnxA1 had the capacity to improve cardiac repair by acting on macrophages in the ischemic myocardium stimulating VEGF-A production.

THERAPEUTIC OVEREXPRESSION OF AnxA1 PREVENTS HEART FAILURE IN PIGS. To challenge the translational potential of our findings, we used cardiotropic adeno-associated viral vectors to overexpress full-length AnxA1 in pig hearts. AnxA1 overexpression was confirmed by immunofluorescence staining in the myocardial tissue of pigs (**Figure 5A**) as well as by in situ hybridization (Online Figure 12). Functionally, overexpression of AnxA1 led to a reduction in infarct size (**Figures 5B to 5D**). Likewise, a trend toward reduced serum cardiac troponin I levels was noted (Online Figure 13A). Consistent with the data obtained in mice, AnxA1 overexpression in pigs counteracted the increase of left ventricular end-diastolic pressure (**Figure 5E**), an important cardiac parameter that positively correlates with the risk of developing heart failure (10). In addition, overexpression of AnxA1 lowered the reduction of ejection fraction after MI (**Figure 5F**). Angiogenesis within the infarct area was also increased after AnxA1 overexpression (**Figure 5G**, Online Figure 13B). In addition, AnxA1 overexpression enhanced the total amount of VEGF-A and, specifically, the number of VEGF-A-expressing cardiac macrophages (**Figures 5H and 5I**), thus corroborating a regulatory loop defined by us in mice. Finally, and in agreement with observations made in mice, we observed no differences in cardiac collagen deposition and the number of apoptotic cells (Online Figures 13C and 13D).

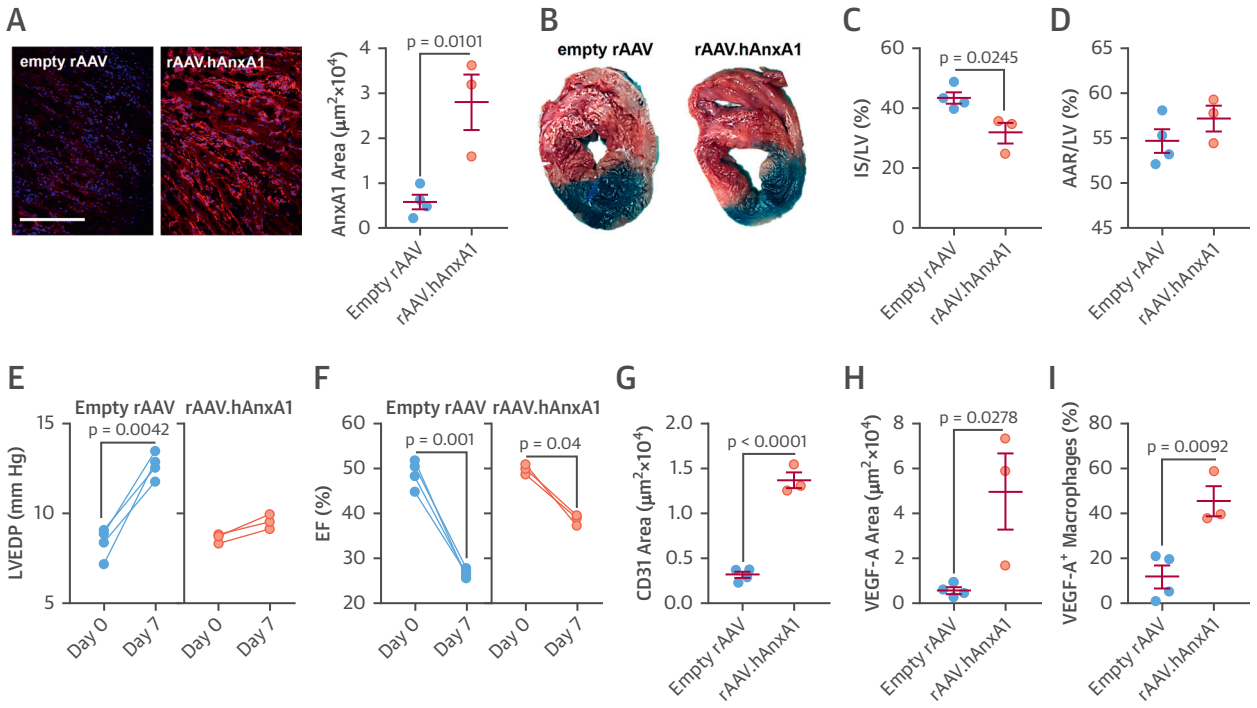
In additional studies, we aimed to link our observations to human pathology. For this purpose, we assessed cardiac AnxA1 expression in pathology samples obtained from patients deceased from acute MI. In these samples, we observed a highly significant correlation between AnxA1 in the infarct area and CD31 staining (Online Figures 14A and 14B). In addition, AnxA1 staining strongly correlated with VEGF-A expression within cardiac macrophages (Online Figure 14C). These data suggest that regulatory mechanisms defined in mice and pigs may also be important in human disease.

DISCUSSION

Despite significant advances in cardiovascular medicine, optimization of myocardial repair and regeneration remain a major therapeutic challenge (11). Heart failure as a consequence of impaired restoration of myocardial functionality is an increasing cause of morbidity and mortality (12,13). Thus, we aimed to connect inflammatory processes initiated early after MI with delayed healing responses. Based on its abundance in myeloid cells and its reported inflammation-resolving and tissue-reparative properties, we chose to focus our studies on AnxA1. Our work provides evidence for the importance of endogenous AnxA1 during cardiac repair following myocardial ischemia (**Central Illustration**). Mechanistically, AnxA1 generates a reparative, proangiogenic macrophage phenotype that controls myocardial neoangiogenesis. Therapeutic delivery or overexpression of AnxA1 in mice or pigs strongly improved cardiac function, thus supporting the translational potential of AnxA1-centered therapy.

Inflammatory cell infiltration in the myocardium plays a crucial role during cardiac injury and repair (14). The initial recruitment of inflammatory cells is a dynamic, well-organized process of a sequential infiltration in the injured myocardium dominated by neutrophils and monocytes. Although inflammation is necessary for debridement after ischemia, extensive inflammation is thought to drive fibrosis. Therefore, the ability to resolve inflammation by the cell populations recruited to the myocardium

FIGURE 5 **Annexin A1 Overexpression in Pigs Improves Cardiac Functionality**

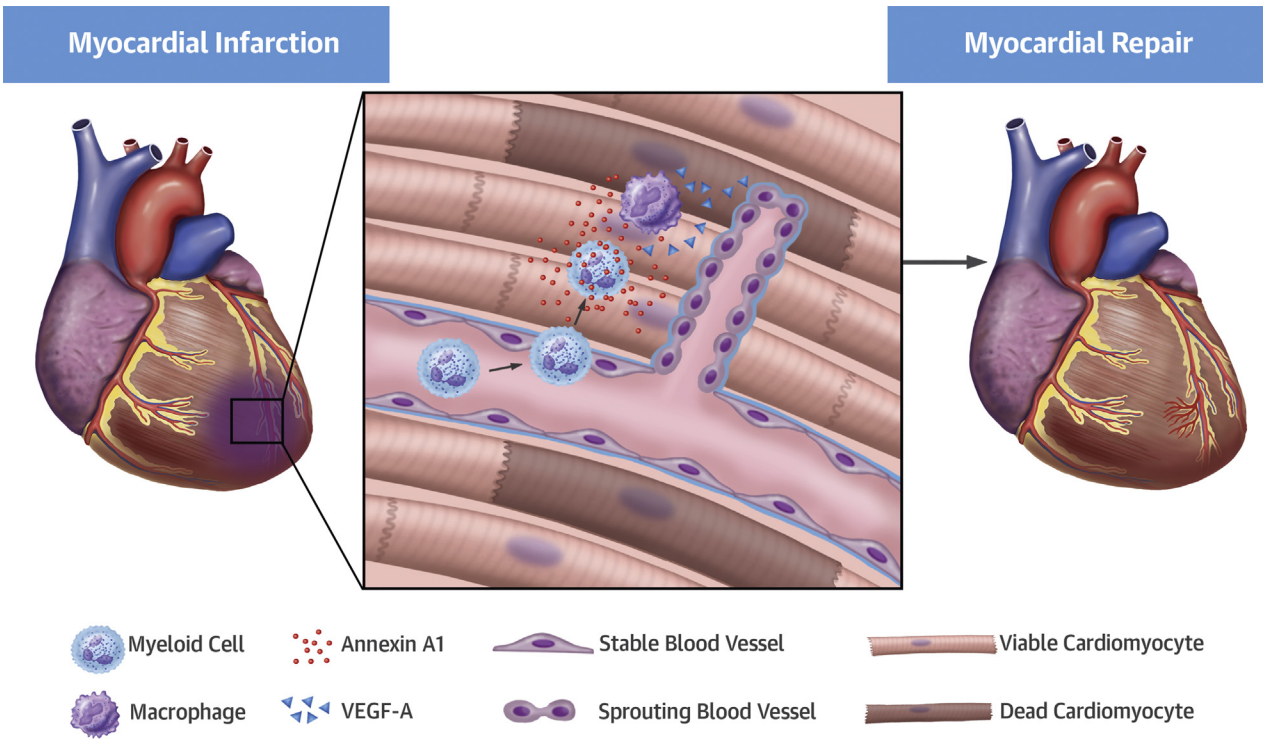


Pigs were treated with an empty adenovirus (empty rAAV) or with an adeno-associated virus (AAV) promoting expression of human AnxA1 (rAAV.hAnxA1) 2 weeks prior to ischemia-reperfusion injury. **(A)** Expression of hAnxA1 in the ischemic area. Representative images are displayed to the left, scale bar, 100 µm. **(B)** Representative images post-MI of infarct size and area at risk in control and rAAV.hAnxA1-treated pigs. **(C and D)** Quantification of infarct size **(C)** and area at risk **(D)**. **(E)** LVEDP measurements before and after ischemia-reperfusion injury. **(F)** Ejection fraction measurements before and after ischemia-reperfusion injury. **(G)** Assessment of myocardial CD31 immunostaining. **(H)** Quantification of myocardial VEGF-A expression. **(I)** Density of cardiac macrophages expressing VEGF-A. All values are mean ± SEM. Following Shapiro-Wilk normality test, Student's *t*-test was used in **A, C, D, and G to I**; Wilcoxon matched-pairs signed rank test was used in **E and F**. Each **dot** represents 1 pig. AAR = area at risk; AnxA1 = annexin A1; EF = ejection fraction; IS = infarct size; LV = left ventricle; LVEDP = left ventricular end-diastolic pressure; rAAV = recombinant adeno-associated virus.

becomes crucial for successful outcome. In particular, cardiac macrophages are considered a potential therapeutic target in promoting myocardial healing facilitating phagocytosis of necrotic cells and angiogenesis (15,16). Pro-reparative macrophages release anti-inflammatory cytokines and angiogenic factors, which are important to resolve the inflammation and promote angiogenesis (17,18). In fact, alternatively activated macrophages have been shown to dictate repair mechanisms post-MI (19). The clinical applicability of such knowledge has been demonstrated in patients receiving alternatively activated macrophages promoting improved cardiac function (20). In line with the concept of endogenous regulatory loops fostering macrophage reprogramming, a recent study revealed that neutrophil-borne secretory products generate a reparative macrophage phenotype, thereby supporting post-MI repair (21). In a similar situation, activation of E-prostanoid 3 receptor in macrophages was able to improve cardiac repair after MI in a process involving VEGF release with consequent improvement of neovascularization in peri-infarct areas (22).

Angiogenesis is a key factor in the process of cardiac healing after MI. While strategies inducing angiogenesis have become a very attractive approach to improve cardiac repair, none have shown sufficient efficacy. As an example, transplantation of autologous endothelial progenitor cells or their pharmacological mobilization showed promising data in animal models, but was largely disappointing in clinical trials (23). The latter is based on low efficacy as well as undesired side-effects, including angiogenic growth at remote sites. Delivery of growth factors such as VEGF may thus be an alternative strategy to facilitate growth of blood flow vessels in failing hearts. However, such approaches

CENTRAL ILLUSTRATION **Annexin A1 Treatment Improves Cardiac Functionality by Driving Macrophage Polarization Toward a Pro-Angiogenic Phenotype**



Ferraro, B. et al. J Am Coll Cardiol. 2019;73(23):2990–3002.

Cardiac repair after MI results from a finely orchestrated series of events, initiated after acute inflammation and immune cell infiltration that serve to digest and clear damaged cells and extracellular matrix. This phase is followed by a reparative phase with resolution of inflammation. Here, we show that myeloid cell-borne Annexin A1 shifts macrophage polarization toward a reparative, pro-angiogenic phenotype that ultimately enhances sprouting of new blood vessels through the release of vascular endothelial growth factor (VEGF)-A, hence improving cardiac functionality.

are hampered by unfavorable pharmacokinetics and biodistribution. In addition, spatio-temporal delivery of therapeutic proteins needs to be very tightly regulated to avoid side effects, such as the promotion of tumor growth or retinopathy. Another important issue in therapeutic angiogenesis is that the delivery of a single growth factor might be insufficient to mimic the complex regulatory mechanisms driving neovascularization. In the case of VEGF, several formulations and delivery strategies have been designed to overcome such problems (24,25). Although some of these strategies appear promising, the overall efficacy remains rather low. Treatment with AnxA1 or its mimetics may overcome some of the shortcomings of VEGF. Its multifaceted activity profile acts at several levels to dampen inflammation while enhancing repair. AnxA1 delivery reduces accumulation of macrophages in models of cardiovascular inflammation (4) and reprograms macrophages toward a reparative phenotype epitomized by a favorable cytokine profile (9,26) and the release of angiogenic growth factors. Overall, the sum of these mechanisms may exert beneficial effects that cannot be matched by delivery of VEGF-A only.

In our study, we identify an endogenous reparatory loop centered on myeloid cell-derived AnxA1 promoting macrophage reprogramming toward an angiogenic phenotype, with VEGF-A being the signature growth factor. Ultimately, this mechanism promotes cardiac repair. Although studies on AnxA1 in the context of angiogenesis are scarce, there is evidence for pro-reparative effects of AnxA1. Consequently, strategies have been developed to deliver AnxA1 into diseased tissue. In the context of cardiac ischemia-reperfusion, Ac2-26 exhibits beneficial effects in part due to an inhibition of neutrophil accumulation (27). In addition, Ac2-26 preserves

the contractile function as well as the viability of cardiomyocytes (28,29). In the context of inflammatory bowel diseases, AnxA1-containing extracellular vesicles released by injured epithelium stimulated epithelial migration and wound healing (6). AnxA1 treatment in patients with rheumatoid arthritis promoted cartilage protection by increasing the production of transforming growth factor-β (30). In atherosclerosis studies, nanoparticles containing Ac2-26 increased lesion size by reducing lesional superoxide and collagenase activity, demonstrating their important role in tissue repair during atherogenesis (26). Such promising findings on the potential therapeutic use of AnxA1 have stimulated interest to design new therapeutic formulations containing AnxA1 or AnxA1 mimetics, such as the controlled-release hydrogels for dermal wound repair application and targeted polymeric nanoparticles containing AnxA1 mimetic peptide Ac2-26 for tissue repair (5,26,31,32). These pharmaceutical strategies offer further benefits, overcoming the critical pharmacokinetics of short peptides in an in vivo scenario, with the advantage of protecting them from proteolysis during pharmacological treatment, and facilitating their delivery to injury sites.

Taken together, AnxA1-based pharmacological strategies could be very effective during cardiac repair. Indeed, AnxA1 contributes to tissue homeostasis by inducing macrophage reprogramming toward a resolving pro-angiogenic phenotype, pointing to AnxA1 as a promising therapeutic agent for treating MI.

STUDY LIMITATIONS. This study delivers a mouse-centered mechanistic assessment of the importance of Annexin A1 in myocardial repair. An Annexin A1-based intervention was tested in mice and pigs. Although the expression and sequence of Annexin A1 is well conserved between species, FPR2, the receptor of Annexin A1, exhibits differences in amino acid sequence and affinity. Hence, data generated here require consolidation before moving toward human intervention. In this context it is also important to point out that all mouse experiments were performed in a model of permanent LAD ligation rather than LAD ischemia-reperfusion. Finally, timing of the Annexin A1 delivery is at the essence as the balance of inflammation and repair is tightly controlled. Hence, further studies on timing, dosage, and tissue-specific delivery are warranted.

CONCLUSIONS

AnxA1 promotes tissue repair in the ischemic myocardium by activating a pro-angiogenesis pathway involving cardiac macrophages and VEGF release.

ACKNOWLEDGMENT The authors thank Olga Schengel for excellent technical assistance.

ADDRESS FOR CORRESPONDENCE: Dr. Oliver Soehnlein, Institute for Cardiovascular Prevention, Ludwig-Maximilians-University Munich, Pettenkoferstr. 9, 80336 Munich, Germany. E-mail: oliver.soehnlein@gmail.com. Twitter: @LMU_Muenchen.

PERSPECTIVES

COMPETENCY IN MEDICAL KNOWLEDGE: Annexin A1 shifts cardiac macrophage activity toward a reparative phenotype that promotes myocardial repair after injury without impairing systemic immunity.

TRANSLATIONAL OUTLOOK: Clinical studies are needed to establish the safety of treatment with AnxA1 after MI and its effect on ventricular function.

REFERENCES

1. Benjamin EJ, Virani SS, Callaway CW, et al. Heart disease and stroke statistics-2018 update: a report from the American Heart Association. Circulation 2018;37:e67–492.

2. Hilgendorf I, Gerhardt LM, Tan TC, et al. Ly-6Chigh monocytes depend on Nr4a1 to balance both inflammatory and reparative phases in the infarcted myocardium. Circ Res 2014;114:1611–22.

3. de Jong R, Leoni G, Drechsler M, Soehnlein O. The advantageous role of annexin A1 in cardiovascular disease. Cell Adh Migr 2017;11:261–74.

4. Drechsler M, de Jong R, Rossaint J, et al. Annexin A1 counteracts chemokine-induced arterial myeloid cell recruitment. Circ Res 2015;116:827–35.

5. Leoni G, Alam A, Neumann PA, et al. Annexin A1, formyl peptide receptor, and NOX1 orchestrate epithelial repair. J Clin Invest 2013;123:443–54.

6. Leoni G, Neumann PA, Kamaly N, et al. Annexin A1-containing extracellular vesicles and polymeric nanoparticles promote epithelial wound repair. J Clin Invest 2015;125:1215–27.

7. Leoni G, Nusrat A. Annexin A1: shifting the balance towards resolution and repair. Biol Chem 2016;397:971–9.

8. Swirski FK, Nahrendorf M. Leukocyte behavior in atherosclerosis, myocardial infarction, and heart failure. Science 2013;339:161–6.

9. Qin CX, Finlayson SB, Al-Sharea A, et al. Endogenous annexin-a1 regulates haematopoietic stem cell mobilisation and inflammatory response post myocardial infarction in mice in vivo. Sci Rep 2017;7:16615.

10. Brienesse SC, Davies AJ, Khan A, Boyle AJ. Prognostic value of LVEDP in acute myocardial infarction: a systematic review and meta-analysis. J Cardiovasc Transl Res 2018;11:33–5.

11. Cahill TJ, Choudhury RP, Riley PR. Heart regeneration and repair after myocardial infarction: translational opportunities for novel therapeutics. Nat Rev Drug Discov 2017;16:699–717.

12. Sanchis-Gomar F, Perez-Quilis C, Leischik R, Lucia A. Epidemiology of coronary heart disease and acute coronary syndrome. Ann Transl Med 2016;4:256.

13. Anderson JL, Morrow DA. Acute Myocardial Infarction. N Engl J Med 2017;376:2053–64.

14. Liehn EA, Postea O, Curaj A, Marx N. Repair after myocardial infarction, between fantasy and reality: the role of chemokines. J Am Coll Cardiol 2011;58:2357–62.

15. Frantz S, Nahrendorf M. Cardiac macrophages and their role in ischaemic heart disease. Cardiovasc Res 2014;102:240–8.

16. Frangogiannis NG. The inflammatory response in myocardial injury, repair, and remodelling. Nat Rev Cardiol 2014;11:255–65.

17. Nahrendorf M, Swirski FK, Aikawa E, et al. The healing myocardium sequentially mobilizes two monocyte subsets with divergent and complementary functions. J Exp Med 2007;204:3037–47.

18. Vannella KM, Wynn TA. Mechanisms of organ injury and repair by macrophages. Annu Rev Physiol 2017;79:593–617.

19. Shiraishi M, Shintani Y, Shintani Y, et al. Alternatively activated macrophages determine repair of the infarcted adult murine heart. J Clin Invest 2016;126:2151–66.

20. Patel AN, Henry TD, Quyyumi AA, et al. Ixmyelocel-T for patients with ischaemic heart failure: a prospective randomised double-blind trial. Lancet 2016;387:2412–21.

21. Horckmans M, Ring L, Duchene J, et al. Neutrophils orchestrate post-myocardial infarction healing by polarizing macrophages towards a reparative phenotype. Eur Heart J 2017;38:187–97.

22. Tang J, Shen Y, Chen G, et al. Activation of E-prostanoid 3 receptor in macrophages facilitates cardiac healing after myocardial infarction. Nat Commun 2017;8:14656.

23. Bianconi V, Sahebkar A, Kovanen P, et al. Endothelial and cardiac progenitor cells for cardiovascular repair: a controversial paradigm in cell therapy. Pharmacol Ther 2018;181:156–68.

24. Oduk Y, Zhu W, Kannappan R, et al. VEGF nanoparticles repair the heart after myocardial infarction. Am J Physiol Heart Circ Physiol 2018;314:H278–84.

25. Rodness J, Mihic A, Miyagi Y, Wu J, Weisel RD, Li RK. VEGF-loaded microsphere patch for local protein delivery to the ischemic heart. Acta Biomater 2016;45:169–81.

26. Fredman G, Kamaly N, Spolitu S, et al. Targeted nanoparticles containing the proresolving peptide Ac2-26 protect against advanced atherosclerosis in hypercholesterolemic mice. Sci Transl Med 2015;7:275ra20.

27. La M, D'Amico M, Bandiera S, et al. Annexin 1 peptides protect against experimental myocardial ischemia-reperfusion: analysis of their mechanism of action. FASEB J 2001;15:2247–56.

28. Ritchie RH, Gordon JM, Woodman OL, Cao AH, Dusting GJ. Annexin-1 peptide Anx-1(2-26) protects adult rat cardiac myocytes from cellular injury induced by simulated ischaemia. Br J Pharmacol 2005;145:495–502.

29. Qin C, Buxton KD, Pepe S, et al. Reperfusion-induced myocardial dysfunction is prevented by endogenous annexin-A1 and its N-terminal-derived peptide Ac-ANX-A1(2-26). Br J Pharmacol 2013;168:238–52.

30. Headland SE, Jones HR, Norling LV, et al. Neutrophil-derived microvesicles enter cartilage and protect the joint in inflammatory arthritis. Sci Transl Med 2015;7:315ra190.

31. Del Gaudio P, De Cicco F, Aquino RP, et al. Evaluation of in situ injectable hydrogels as controlled release device for ANXA1 derived peptide in wound healing. Carbohydr Polym 2015;115:629–35.

32. Kamaly N, Fredman G, Subramanian M, et al. Development and in vivo efficacy of targeted polymeric inflammation-resolving nanoparticles. Proc Natl Acad Sci U S A 2013;110:6506–11.

KEY WORDS annexin A1, cardiac repair, inflammation, myocardial infarct, neovascularization

APPENDIX For an expanded Methods section as well as supplemental figures and videos, please see the online version of this paper.