Targeted Injection of a Biocomposite Material Alters Macrophage and Fibroblast Phenotype and Function following Myocardial Infarction: Relation to Left Ventricular Remodeling

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Received April 23, 2014; accepted July 11, 2014

ABSTRACT

A treatment target for progressive left ventricular (LV) remodeling prevention following myocardial infarction (MI) is to affect structural changes directly within the MI region. One approach is through targeted injection of biocomposite materials, such as calcium hydroxyapatite microspheres (CHAM), into the MI region. In this study, the effects of CHAM injections upon key cell types responsible for the MI remodeling process, the macrophage and fibroblast, were examined. MI was induced in adult pigs before randomization to CHAM injections (20 targeted 0.1-ml injections within MI region) or saline. At 7 or 21 days post-MI ($n = 6$ /time point per group), cardiac magnetic resonance imaging was performed, followed by macrophage and fibroblast isolation. Isolated macrophage profiles for monocyte chemotactic macrophage inflammatory protein-1 as measured by real-time polymerase chain reaction increased at 7 days post-MI in the CHAM

group compared with MI only (16.3 ± 6.6 versus 1.7 ± 0.6 cycle times values, $P < 0.05$), and were similar by 21 days post-MI. Temporal changes in fibroblast function and smooth muscle actin (SMA) expression relative to referent control ($n = 5$) occurred with MI. CHAM induced increases in fibroblast proliferation, migration, and SMA expression—indicative of fibroblast transformation. By 21 days, CHAM reduced LV dilation (diastolic volume: 75 ± 2 versus 97 ± 4 ml) and increased function (ejection fraction: $48 \pm 2\%$ versus $38 \pm 2\%$) compared with MI only (both $P < 0.05$). This study identified that effects on macrophage and fibroblast differentiation occurred with injection of biocomposite material within the MI, which translated into reduced adverse LV remodeling. These unique findings demonstrate that biomaterial injections impart biologic effects upon the MI remodeling process over any biophysical effects.

Introduction

Following a myocardial infarction (MI), changes in the size, shape, and function of the left ventricle (LV) often occur, which are characterized by progressive extension and thinning of the MI region, termed infarct expansion (Ertl and Frantz, 2005; Spinale, 2007; Konstam et al., 2011; Morita et al., 2011; Tous et al., 2012). Specifically, as a function of MI wall thinning, increased radial wall stress and local strain patterns occur, which is further compounded by dyskinesis of the affected region. Although certainly a multifactorial process, these biophysical changes can in turn promote

inflammatory signaling and proteolytic pathways, which can cause a “feed forward” effect on MI expansion and adverse LV remodeling (Dewald et al., 2005; Hohensinner et al., 2010; Frangogiannis, 2012). As such, strategies that can potentially attenuate these adverse mechanical events, such as the injection of biomaterials directly into the MI region, have been identified as a potential therapeutic strategy (Mukherjee et al., 2008; Pilla et al., 2009; Ifkovits et al., 2010; Morita et al., 2011; Rane et al., 2011; Tous et al., 2012; Shuman et al., 2013). One of the prototype biomaterials that has been evaluated in preclinical post-MI models is based on a hydroxyapatite composition (Mukherjee et al., 2008; Morita et al., 2011; Shuman et al., 2013). For example, studies from this laboratory have previously demonstrated that targeted injections of calcium hydroxyapatite microspheres (CHAM) into the MI region will directly reduce local strain patterns and MI thinning/expansion (Mukherjee et al., 2008; Dixon et al., 2011; Morita et al., 2011; Shuman et al., 2013).

This work was supported by the National Institutes of Health National Heart, Lung, and Blood Institute [Grants HL095608, HL111090, and HL063954]; and a Merit Award from the Veterans' Affairs Health Administration. J.A.B. was supported by a National Institutes of Health grant [Grant T32-HL007954].

dx.doi.org/10.1124/jpet.114.215798.

ABBREVIATIONS: ANOVA, analysis of variance; CCR2, C-C chemokine receptor type 2; CHAM, calcium hydroxyapatite microspheres; Ct, cycle times; ECM, extracellular matrix; IL-8, interleukin-8; LTBP-1, latency binding protein-1; LV, left ventricular; MCP-1, monocyte chemotactic protein-1; MI, myocardial infarction; MIP-1A, macrophage inflammatory protein-1 α ; MMP, matrix metalloproteinase; MRI, magnetic resonance imaging; PCR, polymerase chain reaction; SMA, smooth muscle actin; TGF, transforming growth factor β ; TNF, tumor necrosis factor α .

Second, CHAM injections within the MI region have been shown to favorably affect indices of extracellular matrix (ECM) remodeling, and as such, favorably alter local stress patterns within the MI region (Dixon et al., 2011; Morita et al., 2011). Although these studies identified that biocomposite material injections favorably affect post-MI remodeling, what remains poorly understood is how these materials may affect endogenous cell types within the MI region. Pivotal cellular events in the post-MI remodeling process include changes in the phenotype and function of the macrophage and fibroblast (Tomasek et al., 2002; Chapman et al., 2003; Dewald et al., 2005; Lindsey et al., 2005; Hohensinner et al., 2010; Baum and Duffy, 2011; Widgerow, 2011; Frangogiannis, 2012; Goldsmith et al., 2013). Specifically, a shift in the expression profile of macrophages, termed macrophage polarization, occurs in the post-MI period, which can affect post-MI remodeling (Brown et al., 2009; Anzai et al., 2012; Frangogiannis, 2012; Ma et al., 2013). The proliferation of fibroblasts with a more smooth muscle-like phenotype occurs within the MI region, and this process has been termed fibroblast transdifferentiation, and these fibroblasts termed myofibroblasts (Tomasek et al., 2002; Spinale, 2007; Goldsmith et al., 2013). The purpose of this study was to define the effects of targeted CHAM injections within the MI region on determinants of ECM remodeling, and more importantly upon macrophage and fibroblast phenotype, and relate these changes to the LV remodeling process.

Materials and Methods

Overview and Rationale. An adult pig model of post-MI remodeling was used whereby injections of CHAM (carboxymethylcellulose, Radiess; Bioform Medical Inc., San Mateo, CA) were performed at the time of MI induction (Dixon et al., 2011; Morita et al., 2011). CHAM has been used clinically as a dermal filler for facial augmentation whereby the CHAM microspheres (25–45 μ m diameter) are suspended in a gel carrier composed of glycerin (Ahn, 2007; Jacovella, 2008). The disposition of CHAM following injections is that of a bioceramic, whereby the gel carrier degrades quickly and is then followed by a more prolonged cell-dependent biodegradable process of the microspheres (Ahn, 2007; Jacovella, 2008; Dixon et al., 2011; Tous et al., 2012; Shuman et al., 2013). More specifically, CHAM is cleared by a macrophage-dependent process, which is then accompanied by proliferation of fibroblasts (Shumaker et al., 2006; Ahn, 2007; Jacovella, 2008; Dixon et al., 2011; Tous et al., 2012). These cell types, the macrophage and fibroblast, play a critical role in the post-MI remodeling process (Lindsey et al., 2001; Ertl and Frantz, 2005; Hohensinner et al., 2010; Frangogiannis, 2012; Freytes et al., 2013; Goldsmith et al., 2013). Past studies have provided functional evidence that the effects of injected acellular biomaterials, such as CHAM upon LV geometry and post-MI remodeling, are not entirely due to the biophysical effects of the material itself but rather to the localized cellular response evoked by these biomaterials (Ifkovits et al., 2010; Rane et al., 2011; Tous et al., 2011, 2012; Burdick et al., 2013; Shuman et al., 2013). Therefore, understanding how macrophage and fibroblast form and function within the MI region are affected following targeted CHAM injections remains a critical issue if these biomaterials are to continue advancement as a possible therapeutic for post-MI remodeling. Past studies have identified that critical time points of post-MI remodeling in terms of infarct expansion and structural changes within the MI region occur at 7 and 21 days post-MI (Ertl and Frantz, 2005; Spinale, 2007; Konstam et al., 2011; Frangogiannis, 2012). Accordingly, LV geometry and function, indices of ECM remodeling, as well as studies of isolated macrophages and fibroblasts were performed at these post-MI time points to define specific phenotypic changes in these cell types with respect to CHAM injections.

Pig MI Model and CHAM Injections. This laboratory has developed a reproducible method for inducing MI in adult pigs and a pig MI model was used in the present study (Dixon et al., 2010; Frangogiannis, 2012). In brief, pigs ($n = 24$, Yorkshire, 25 kg) were anesthetized with isoflurane (2.5%), and through a left thoracotomy, the LV free wall encompassing the circumflex coronary artery was exposed. The obtuse marginals-1 and -2 were ligated, creating an MI of approximately 15–18% of the LV at risk (Dixon et al., 2010; Frangogiannis, 2012). Platinum markers (10, 2 mm) were sutured around the periphery of the MI region to allow for infarct localization during subsequent magnetic resonance imaging (MRI) studies. Pigs were then randomized to undergo CHAM injections within the MI region or saline injections (MI controls) at the time of MI induction. Using the sutured platinum markers as a guide for the MI region, 20 evenly spaced CHAM injections over the entire MI region were performed. Each injection was performed using a 27-gauge needle placed into the mid-myocardial wall whereby 150 μ l of CHAM was injected at each of these 20 sites, thus resulting in a total volume of 3 ml of CHAM injected within the mid-myocardium of the MI region. Due to the semisolid cohesive nature of the CHAM formulation, the injected material did not egress from the targeted injection site due to myocardial motion/compression. Pigs were then randomized to undergo terminal MRI studies and cell isolation at 7 or 21 days post-MI (6 pigs/group per time point). A set of age-matched referent controls (no MI, $n = 5$) were also included. All animals were treated and cared for in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (eighth edition, Washington, DC, 2011), and all protocols were approved by the University of Pennsylvania Institutional Animal Care and Use Committee.

Cardiac MRI Studies. The pigs were anesthetized with isoflurane (2.5%) and maintained under general anesthesia during the MRI studies. A high-fidelity pressure transduction catheter (Millar Instruments, Houston, TX) was positioned for the purposes of cardiac cycle gating. MRI was performed using a 3T Siemens Trio A Tim Magnetom scanner (Siemens, Malvern, PA). Animals underwent prospectively gated three-dimensional steady-state free precession cine MRI for volumetric analysis using the following parameters: field of view: 300×244 , acquisition matrix: 192×156 , repetition time: 3.11 milliseconds, echo time: 1.53 milliseconds, bandwidth: 1184 Hz/pixel, slice thickness: 4 mm, averages: 2. Fifteen minutes following intravenous injection of 0.1 mmol/kg gadobenate dimeglumine (MultiHance; Bracco Diagnostics, Princeton, NJ), infarcts were visualized using a three-dimensional late gadolinium-enhanced spoiled gradient echo sequence with the following parameters: field of view: 350×350 , acquisition matrix: 256×256 , repetition time: 591.28 milliseconds, echo time: 2.96 milliseconds, inversion time: 200–300 milliseconds, flip angle: 25°, averages: 2. Imaging data sets were blinded to the end user throughout postprocessing. LV volume and global function data were obtained using prospective steady-state free precession cine MRI images (Yushkevich et al., 2006). Raw short-axis images were automatically sorted, cropped, and contrast normalized in a custom Matlab (MathWorks, Natick, MA) program to ensure homogenous LV coverage and image quality, respectively. Segmentation was then performed through all cardiac phases of the sorted and correct images using a semiautomated three-dimensional active contour segmentation program (ITK-SNAP, open access/source). LV end-diastolic volume and ejection fraction were then computed throughout the entire cardiac cycle from segmented images using in-plane and through-plane spatial resolution information. LV wall thickness at the site of the MI was also computed at end-diastole.

Following the MRI studies, and maintaining a surgical plane of anesthesia, the LV was harvested, and the MI region was sectioned into equal thirds and then prepared for measurements of ECM remodeling as well as macrophage and fibroblast isolation.

Determinants of ECM Remodeling. Full-thickness sections of the MI region were prepared for RNA isolation and histology using methods described previously (Mukherjee et al., 2008; Dixon et al., 2010, 2011). For RNA extraction, MI samples were placed in an initial RNA extraction solution (RNA Later; Life Technologies, Grand

Island, NY) and then fully extracted (RNeasy Fibrous Tissue Mini Kit; Qiagen, Valencia, CA), whereby the quantity and quality of the RNA was determined (Experion Automated Electrophoresis System; Bio-Rad Laboratories, Hercules, CA). RNA (1 μ g) was reverse transcribed to generate cDNA (iScript cDNA Synthesis Kit; Bio-Rad). The cDNA was amplified with gene/pig-specific primer/probe sets (RT² Profiler PCR Custom Array; Qiagen) corresponding to determinants of ECM synthesis and degradation: collagen type-1, transforming growth factor β (TGF), latency binding protein-1 for TGF (LTBP-1), matrix metalloproteinase-2 (MMP-2), matrix metalloproteinase-9 (MMP-9), and membrane-type MMP-1 (MMP-14). The array was designed to contain primers for each gene of interest along with internal controls and contamination controls. The reaction was performed (RT² SYBR Green qPCR Mastermix; Qiagen) and quantified by real time (CFX96 real-time PCR detection system; Bio-Rad). The real-time polymerase chain reaction (PCR) fluorescence signal was converted to cycle times (Ct) normalized to glyceraldehyde-3-phosphate dehydrogenase (Δ Ct) and final results expressed as $2^{-\Delta\text{Ct}} \times 10^3$. All PCR assays were performed in duplicate.

The formalin-fixed full-thickness MI samples were embedded, sectioned (7 μ m), and stained with Picro-sirius red for fibrillar collagen, and the percent area of collagen within the remote and MI regions was computed using computer-assisted morphometry (Mukherjee et al., 2008; Dixon et al., 2011). Immunostaining was used to colocalize with cells that stained positive for α -smooth muscle actin (1:100; α -smooth muscle actin; Sigma-Aldrich, St. Louis, MO), a marker for actin present in vascular smooth muscle cells as well as myofibroblasts, using approaches described previously (Lindsey et al., 2005; Dixon et al., 2011).

Macrophage Isolation. Macrophages were isolated from the MI region using methods detailed previously (Brown et al., 2009; Freytes et al., 2013; Ma et al., 2013). In brief, the MI samples (500 mg) were minced and incubated in a digestion solution (Liberase Blendzyme, 0.25 mg/ml; 37°C; Roche, Indianapolis, IN) with gentle trituration for 60 minutes, resuspended in cold buffer [protein extraction buffer: bovine serum albumin, phosphate-buffered saline, autoMACS rinsing solution (Miltenyi Biotec, San Diego, CA)], and centrifuged (300g, 10 minutes) with these last steps repeated ($\times 3$). The suspension was filtered (Miltenyi Biotec) and cell density determined (hemocytometry), diluted to 2×10^8 cells/ml, and the cell suspension incubated with CD11b microbeads (Miltenyi Biotec), run over a magnetic separation column (Mini MACS separator; Miltenyi Biotec), and rinsed with protein extraction buffer. The retained CD11b cells (macrophages) were eluted into culture media (Dulbecco's modified Eagle's medium) and placed into aliquots at a final concentration of $2 \times 10^7/100 \mu$ l. RNA was isolated from these macrophages as described in the previous paragraph, and PCR was performed for cytokine expression of porcine-specific tumor necrosis factor α (TNF), interleukin-8 (IL-8), monocyte chemoattractant protein-1 (MCP-1), and macrophage inflammatory protein-1 α (MIP-1A).

Fibroblast Isolation. Fibroblasts from the MI region were isolated using selective collagenase digestion as described previously (Chapman et al., 2003; Lindsey et al., 2005). Fibroblasts were maintained in tissue culture flasks (150 cm²; Falcon, Fisher Scientific, Pittsburgh, PA) with complete growth media (Fibroblast Growth Medium; PromoCell, Heidelberg, Germany) containing 17% fetal bovine serum (Invitrogen, Carlsbad, CA) and Fibroblast Growth Supplement (PromoCell). The cells were incubated under standard culture conditions in a humidified incubator at 37°C, 5% CO₂ (21% O₂). The confluent myocardial fibroblast cultures from passages 3–5 were used whereby parallel groups were studied from each time point. Fibroblast proliferation was determined by plating a defined number of cells (30,000/ml) and then determining cell density at 12, 24, and 36 hours (CyQuant Direct Cell Proliferation Assay kit; Life Technologies, Invitrogen). Two-dimensional assays of migration were performed whereby fibroblasts of equivalent density (50,000 cells/ml) were plated in a 24-well formatted cell insert array and then placed in a dual chamber system separated by a polyethylene terephthalate membrane (FluoroBlok; BD Biosciences, San Jose, CA) and incubated for 24 hours. Fibroblast migration into the bottom chamber was determined by fluorescence (CyQuant).

Data Analysis. The treatment assignments were predetermined and each animal was assigned a code number, which was not broken until the completion of the full protocol. Thus, the LV function, PCR, histology, and cell measurements were performed in a blinded fashion. Statistical analyses were performed using STATA statistical software (StataCorp, College Station, TX). LV geometry, function, myocardial PCR, and histology measurements were compared between groups and across time using a two-way analysis of variance (ANOVA). Post-hoc separation following ANOVA was performed using pairwise comparisons with a Bonferroni analysis (prcomp module, STATA). The initial macrophage isolations from the referent control group were extremely low density and thus were not used in this analysis. Accordingly, a one-way ANOVA between the MI and MI/CHAM groups was performed followed by a post-hoc separation Bonferroni analysis. Results are presented as the mean $\pm$ S.E.M., and values of $P < 0.05$ were considered to be statistically significant.

Results

LV Geometry and Function. Representative MRI images for a referent control and at 21 days post-MI for MI only (saline injection) and MI/CHAM are shown in Fig. 1. LV dilation and MI wall thinning were evident in both MI groups but appeared to be attenuated with CHAM injection. LV end-diastolic volume was increased and LV ejection fraction reduced when compared with referent normal values. However, at 21 days post-MI, the magnitude of LV dilation and reduction in function was attenuated in the MI/CHAM group (Fig. 1). Thus, consistent with past reports, targeted CHAM injection within the MI region favorably altered the course of adverse LV remodeling post-MI (Dixon et al., 2011; Morita et al., 2011).

Myocardial PCR and Histology. Quantitative PCR was performed for key indices of ECM remodeling and revealed distinct time-dependent changes in these expression profiles with CHAM injection (Fig. 2). Specifically, indices of ECM degradation such as MMP-2, MMP-9, and MMP-14 were reduced in the MI/CHAM group at 7 days post-MI. MMP-9 mRNA levels remained lower in the MI/CHAM group at 21 days post-MI. Indices of ECM synthesis, such as fibrillar collagen type I, TGF, and LTBP-1, were increased in both MI groups at 7 days post-MI, but collagen type I and LTBP-1 mRNA levels were lower in the MI/CHAM group. Using Picro-sirius red staining, a clear fibrotic pattern was observed in all post-MI groups (Fig. 3), and the density of the collagen weave appeared increased around the sites of CHAM injection. However, absolute values for collagen content were similar between MI groups. A robust increase in smooth muscle actin (SMA) staining was observed in fibroblasts from both MI groups (Fig. 3), and quantitative measurements revealed higher SMA staining at 7 days post-MI in the MI/CHAM group.

Macrophage Phenotype and Fibroblast Function. Quantitative PCR was performed on isolated macrophages in both MI groups (Fig. 4) and revealed that mRNA levels for TNF, IL-8, MCP-1, and MIP-1A were increased in the MI/CHAM group at 7 days post-MI. In isolated fibroblasts from the MI region, both proliferation and migration were higher in the MI/CHAM group when compared with the MI only group at 7 days post-MI (Fig. 5).

Discussion

Strategies to modify stress/strain patterns post-MI utilizing mechanical restraint have been performed at both the global and regional level (Mukherjee et al., 2008; Pilla et al., 2009;

Fig. 1. Cardiac MRI was performed in post-MI pigs at 7 and 21 days post-MI as well as in referent controls. Representative short axis images at end-diastole are shown for a referent control and at 21 days for MI only and MI/CHAM. LV dilation was evident in both MI groups, but at the site of the saline or CHAM injections, a noticeable increase in MI thickness was observed (arrows). LV end diastolic volume was increased in both MI groups compared with referent normal values (54 ± 6 ml, $P < 0.05$) at 7 days with no difference between groups. However, by 21 days post-MI, LV end diastolic volume had increased further in the MI-only group but was attenuated in the MI/CHAM group. LV ejection fraction was reduced in both MI groups at 7 days post-MI from referent control ($56 \pm 5\%$, $P < 0.05$) but was increased from MI-only values in the MI/CHAM group by 21 days post-MI. At 21 days post-MI, LV wall thickness at end-diastole, measured across the MI region (arrows), was increased from MI only values in the MI/CHAM group and was similar to referent normal values (7.35 ± 0.25 mm). (Sample sizes: MI-only: $n = 6$ /time point; MI/CHAM: $n = 6$ /time point; referent normal controls: $n = 5$; $^+P < 0.05$ versus respective MI only values.)

Ifkovits et al., 2010; Morita et al., 2011; Rane et al., 2011; Tous et al., 2012; Shuman et al., 2013). Specifically, a number of past studies using either a hydroxyapatite- or alginate-based biomaterial have demonstrated favorable effects on LV geometry and function post-MI (Mukherjee et al., 2008; Dixon et al., 2011; Morita et al., 2011; Tous et al., 2012; Shuman et al., 2013). These effects included reduced LV dilation, improved pump function, and increased thickness of the MI segment—all observations confirmed in the present study. However, what remained unclear is how biomaterial injections, specifically CHAM injections into the MI region, would alter critical biochemical and cellular determinants of the post-MI remodeling process. The unique findings from the present study were 2-fold. First, in the early post-MI period (7 days), CHAM injections reduced mRNA levels for MMP types, which have been implicated in contributing to adverse post-MI remodeling (Dixon et al., 2011). Second, at this early post-MI time point, CHAM injections increased macrophage cytokine expression as well as fibroblast proliferation and migration rates. Thus, in addition to the biophysical effects of biomaterial injections into the MI region, this study demonstrated, for the first time, time-dependent effects on biochemical/cellular pathways relevant to ECM remodeling which likely contribute to the effects of these materials on the post-MI remodeling process.

Effects of CHAM on Myocardial Wound Healing. The canonical wound healing process, generally applicable with respect to an MI, occurs in three overlapping phases: inflammation,

proliferation, and maturation. The first phase is the acute period in which reactive oxygen species, bioactive signaling molecules, and peptides released from the local environment cause inflammatory cell recruitment and invasion as well as the induction of ECM proteases, such as MMPs (Dewald et al., 2005; Spinale, 2007; Hohensinner et al., 2010; Morita et al., 2011; Frangogiannis, 2012; Tous et al., 2012). The second phase is heralded by proliferation and transdifferentiation of myocardial fibroblasts into myofibroblasts (Tomasek et al., 2002; Baum and Duffy, 2011; Widgerow, 2011; Goldsmith et al., 2013). Further, induction of ECM structural proteins occurs, such as fibrillar collagens, mediated in part by profibrotic signaling molecules such as TGF. The induction of myofibroblasts coupled to ECM-integrin-cytoskeletal interactions can facilitate initial contraction of the wound. The third phase of the wound healing process normally results in complete contraction of the wound, apoptosis of the myofibroblasts, and the formation of a relatively acellular scar. However, in the context of post-MI remodeling, this canonical set of events does not necessarily occur. Rather, there is a continued expression of inflammatory molecules and cells, such as macrophages (Frangogiannis, 2012; Ma et al., 2013). Although a robust proliferation and transdifferentiation of fibroblasts to myofibroblasts occurs within the MI region, this does not result in wound contraction (Ertl and Frantz, 2005; Spinale, 2007; Morita et al., 2011). This is due in part to a shift in matrix proliferative/degradative pathways within these transformed cells. Specifically,

Fig. 2. Quantitative PCR was performed on determinants of ECM remodeling and identified time-dependent changes in mRNA profiles with CHAM injections. At 7 days post-MI, comparative MMP-2, MMP-9, and MMP-14 mRNA levels were reduced in the MI/CHAM group compared with MI-only, and MMP-9 mRNA levels remained reduced from MI-only values at 21 days post-MI. Collagen type-1 (COL1a1) and LTBP-1 mRNA levels were lower in the MI CHAM group at 7 days post-MI. All results are expressed as $2^{-\Delta Ct} \times 10^3$. (Sample sizes: MI-only, $n = 6$ /time point; MI/CHAM, $n = 6$ /time point; referent normal controls, $n = 5$; * $P < 0.05$ versus referent control; + $P < 0.05$ versus respective MI-only; # $P < 0.05$ versus week 1 values.)

the relative expression of the ECM proteolytic enzymes, the MMPs, is amplified and can increase proteolysis of the newly synthesized and immature collagen fibrils within the MI. As a consequence, the continued proliferation and altered expression of the myofibroblasts within the MI region may actually contribute to ECM instability and infarct expansion, ultimately leading to adverse LV remodeling. The present study demonstrated, for the first time, that targeted injection of CHAM within the MI region altered key determinants of the wound healing response. Specifically, at 7 days post-MI, which is a key transition

time point from the acute to more chronic phases of wound healing, MMP expression was attenuated, a shift in macrophage cytokine expression patterns was observed, and a more robust transdifferentiation of fibroblasts occurred with CHAM injection. The summation of these biochemical and cellular effects would in turn cause increased stability of the ECM within the MI region and attenuate infarct expansion.

Effects of CHAM on Matrix Proteases and Macrophages Post-MI. In the present study, CHAM injections reduced the expression of prototypical MMP types that have

Fig. 3. Histologic staining for fibrillar collagen with Picro-sirius red (PSR) revealed a denser collagen weave within the MI region in the CHAM injection group at 7 days post-MI, particularly at the injection sites (open spaces). However, total collagen content was similar in both MI groups. Positive SMA staining in referent normal myocardium was localized to vasculature, particularly small arterioles. However, in the MI regions, SMA staining was positive in spindle-shaped interstitial cells, consistent with a myofibroblast phenotype. SMA staining was increased in the MI/CHAM group at 7 days post-MI when compared with MI-only values. Original image magnification, 40 $\times$. (Sample sizes: MI-only, $n = 6$ /time point; MI/CHAM, $n = 6$ /time point; referent normal controls, $n = 5$; * $P < 0.05$ versus referent control; + $P < 0.05$ versus respective MI-only values.)

Fig. 4. Isolated macrophage cytokine mRNA levels for TNF, IL-8, MCP-1, and MIP-1A were differentially affected within the MI region with CHAM injections, whereby mRNA levels were increased in the MI/CHAM group at 7 days post-MI. Final results expressed as $2^{-\Delta C_t} \times 10^3$. Note: controls were not included due to low yield of macrophages obtained from referent normal myocardial samples. (Sample sizes: MI-only, $n = 6$ /time point; MI/CHAM, $n = 6$ /time point; ⁺ $P < 0.05$ versus respective MI-only; [#] $P < 0.05$ versus week 1 values.)

been implicated in causing adverse LV remodeling post-MI (Spinale, 2007; Spinale et al., 2013). For example, in transgenic constructs, deletion of MMP-9, MMP-2, or MMP-14 have been shown to favorably affect the post-MI remodeling process (Spinale, 2007; Spinale et al., 2013). Thus, the reduction in these specific MMP types with CHAM injections likely reduced overall ECM proteolysis. Neutrophils are one of the major sources of MMP-9 and thus the early induction of MMP-9 in both humans and animals following MI is likely from this inflammatory cell type (Lindsey et al., 2001; Webb et al., 2006; Spinale, 2007). Indeed, the acute magnitude of MMP-9 release has been associated with worsening post-MI remodeling in patients (Webb et al., 2006). Although CHAM injections may have modified neutrophil-mediated expression of MMP-9, this biomaterial did alter another key inflammatory cell type—the macrophage. The relative maturation of the macrophage has been defined by categorical polarization M1 or M2, which change in a time-dependent manner post-MI (Brown et al., 2009; Anzai et al., 2012; Freytes et al., 2013; Ma et al., 2013). In the present study, isolated macrophage expression profiles indicated a greater shift in the M1 phase, as indicated by increased TNF and IL-8 (Brown et al., 2009; Anzai et al., 2012; Ma et al., 2013). Moreover, CHAM injections induced key factors in macrophage function, such as MCP-1 and MIP-1A, which can directly influence post-MI remodeling (Dewald et al., 2005; Anzai et al., 2012). Taken together, these observations would suggest that CHAM injections within the MI region selectively altered the pattern of inflammatory response in the early post-MI period.

In the present study, CHAM injections caused an increased macrophage expression of TNF and IL-8 at an early post-MI time point, which dissipated by 21 days post-MI when compared with MI-only macrophage isolates. In addition, there was a robust early increase in MCP-1 and MIP-1A in macrophages

isolated from the CHAM-injected MI region, which was also reduced by 21 days post-MI. Increased number and activity of macrophages is an essential component of the early wound healing response, whereby the early MI is no exception (Ertl and Frantz, 2005; Lambert et al., 2008; Frangogiannis, 2012; Nahrendorf and Swirski, 2013). While remaining speculative, the increased induction of the macrophage M1 phenotype provoked by CHAM injections in the early post-MI period may have contributed to an acceleration of the early wound healing response. However, even this speculative interpretation may be oversimplistic. There appear to be several macrophage subtypes within the myocardium, and consequently, a much greater heterogeneity of macrophages exists; thus, this polarization classification may be too general (Frangogiannis, 2012; Nahrendorf and Swirski, 2013; Cohen and Mosser, 2014; Epelman et al., 2014). For example, using genetic fate mapping and angiotensin-II infusions in mice, it was demonstrated that unique subpopulations of resident macrophages exist within the myocardium, notably the MCP-1 receptor [C-C chemokine receptor type 2 (CCR2)]-null subtype and a CCR2-positive subtype (Epelman et al., 2014). These CCR2 macrophage subpopulations are likely to play distinctly different roles in terms of inflammation and phagocytosis (Cohen and Mosser, 2014). For example, the CCR2-positive macrophage subtype is efficient in phagocytosis but has diminished capacity to activate T cells (Epelman et al., 2014). The present study demonstrated an induction of MCP-1 in macrophage isolates with CHAM injections early post-MI, which was paralleled by a more than 3-fold reduction in MMP-9 expression, predominantly expressed in activated lymphocytes. Although associative, these findings would suggest that a shift in the subpopulations of macrophages resident within the MI was induced by CHAM injections. Considering these observations and the recent findings that a diverse set of myocardial macrophage subpopulations exist and influence local response to myocardial

Fig. 5. Fibroblasts isolated from the MI region were analyzed with respect to proliferation and migration rates and compared with referent normal myocardial fibroblasts. Fibroblast proliferation and migration rates were higher at 7 days post-MI in the MI/CHAM group. (Sample sizes: MI-only, $n = 6$ /time point; MI/CHAM, $n = 6$ /time point; referent normal controls, $n = 5$; * $P < 0.05$ versus referent control; * $P < 0.05$ versus respective MI-only; # $P < 0.05$ versus week 1 values.)

stress (Cohen and Mosser, 2014; Epelman et al., 2014), then future studies that more carefully phenotype these macrophage subpopulations with MI and CHAM injections would be warranted. In addition, although the present study provides evidence that CHAM injections likely altered macrophage phenotype within the MI region, the potential source and regional distribution of these macrophages was not examined. In both clinical and animal models, it has been demonstrated that the spleen serves as an important source of macrophages (Ismahil et al., 2014; van der Laan et al., 2014). For example, in a mouse model of MI, a concomitant splenectomy altered the magnitude and type of macrophage infiltrating the MI region (Ismahil et al., 2014). The present study focused on macrophages isolated strictly from the MI region to focus on the effects of CHAM injections. Thus, whether and to what degree macrophages from other LV regions, particularly the perfused border zone surrounding the MI region, were affected by CHAM injections remains to be determined.

Myocardial Fibroblast Phenotype Post-MI: Effects of CHAM Injection. Although past studies have identified that the transdifferentiation of fibroblasts to myofibroblasts is a well established cellular event following MI, the relative contribution of these transformed cells in terms of contributing to infarct expansion and ECM remodeling remains an

area of active investigation (Tomasek et al., 2002; Baum and Duffy, 2011; Widgerow, 2011; Goldsmith et al., 2013). In addition, identification of the specific myofibroblast phenotype(s) within the post-MI context can be problematic (Tomasek et al., 2002; Goldsmith et al., 2013). Nonetheless, the expression of SMA within fibroblasts has been used to identify the myofibroblast phenotype (Lindsey et al., 2005; Baum and Duffy, 2011; Dixon et al., 2011) and was used in the present study. In the early post-MI period, CHAM injection caused a more robust SMA staining pattern within the MI region, and thus would suggest an increased fibroblast transdifferentiation to myofibroblasts. Moreover, a unique aspect of this study, fibroblasts were isolated from the MI region and subjected to quantifiable measures of proliferation and migration. These measurements clearly identified that CHAM injections within the MI region altered fibroblast proliferation and migration, consistent with a phenotype switch to myofibroblasts (Lindsey et al., 2005; Widgerow, 2011; Goldsmith et al., 2013). This early induction of the myofibroblast phenotype with CHAM injections likely contributed to the attenuation of adverse LV remodeling, such as dilation and thinning of the MI wall. Specifically, myofibroblasts represent a more contractile phenotype and thus would generate local forces within the ECM, which in turn would attenuate the effects of local strain within the MI and thus reduce a stimulus for infarct expansion. Interestingly, this early induction of the myofibroblast phenotype with CHAM injections was not associated with a more robust profibrotic response. Specifically, TGF expression was unchanged with CHAM injections, and LTBP-1 (critical for release of active TGF) and fibrillar collagen type I expression were reduced. Past studies have identified that a key step in TGF signaling is the proteolytic processing of LTBP-1 by MMP-14 (Spinale et al., 2013). Thus, the reduced expression of MMP-14 in the early post-MI period with CHAM injections may not have only reduced ECM proteolysis but also affected the profibrotic response. Indeed, histologic quantification of fibrillar collagen was unchanged with CHAM injection, which further supports that this biomaterial stabilizes the ECM through reduced turnover rather than a profibrotic response.

Summary, Limitations, and Clinical Implications.

Although the basis for infarct expansion is likely to be multifactorial, local mechanical signals, such as increased stress and strain within the MI and border zones, likely promulgate this process. The injection of biomaterials within the MI region have been clearly shown to favorably alter these biomechanical factors and reduce adverse post-MI remodeling, and as such, translational studies regarding the underlying mechanisms and pathways by which these materials are operative have accelerated (Landa et al., 2008; Mukherjee et al., 2008; Pilla et al., 2009; Ifkovits et al., 2010; Morita et al., 2011; Rane et al., 2011; Tous et al., 2011, 2012; Burdick et al., 2013; Johnson and Christman, 2013; Shuman et al., 2013). Using myocardial injections of a hyaluronic acid and poly (lactide-co-glycolide)-based microsphere (~50 μ m) formulation, it has been previously demonstrated that changing the degradation rates of the microsphere formulation (hydroxyethyl methacrylate) or the microsphere concentration (0–300 mg/ml) can be achieved, which in turn can alter tissue mechanical properties (Ifkovits et al., 2010; Tous et al., 2011, 2012). Using a microsphere concentration of 75 mg/ml in this hyaluronic-hydroxyethyl methacrylate formulation resulted in a reduction in LV dilation post-MI (Tous et al., 2012). CHAM, which contains a microsphere size and concentration (Ahn, 2007; Jacovella,

2008) similar to this past study, has also been shown to reduce LV dilation in an ovine model for up to 8 weeks postinjection (Morita et al., 2011). The present study moves the field forward by demonstrating that biomaterial injections also significantly affect critical determinants of inflammation, macrophage maturation, and fibroblast phenotype. These findings provide direct evidence that biomaterials, such as CHAM, can affect the post-MI remodeling process over and above mechanical effects upon the MI region.

The selection of CHAM for the present study was predicated upon past evidence in large animal models that injections of this microsphere formulation were effective in attenuating the post-MI remodeling process (Dixon et al., 2011; Morita et al., 2011), and that this specific formulation has significant clinical exposure, thus holding potential translational relevance (Ahn, 2007; Jacovella, 2008). Since this is a standardized formulation, then whether and to what degree a higher concentration of microspheres or other degradable carriers would affect post-MI remodeling, and more specifically the biologic response variables measured in the present study, remains unknown. Using a porcine model of MI very similar to that of the present study, it was demonstrated that delayed injection (7 days post-MI) of a calcium-alginate formulation could effectively attenuate progressive LV dilation (Mukherjee et al., 2008). In a rat post-MI model, delayed injection of a calcium-alginate formulation was shown to improve LV pump function and reduce dilation when compared with noninjected post-MI animals (Landa et al., 2008). The present study performed the CHAM injections at the time of MI induction, and thus whether and to what degree delayed injections following MI would affect myocardial remodeling remains to be established. The present study as well as the majority of past reports have used an intraoperative myocardial injection method to deliver the biomaterial to the MI region (Landa et al., 2008; Mukherjee et al., 2008; Pilla et al., 2009; Dixon et al., 2011; Morita et al., 2011; Rane et al., 2011; Tous et al., 2012). Although this approach provides for specificity in terms of targeted injections in a reproducible pattern to the MI region, less invasive approaches would be desirable, such as catheter-based methods (Burdick et al., 2013; Johnson and Christman, 2013; Shuman et al., 2013). The design of biomaterial formulations that "self-assemble" following release from a catheter is an area of active research and would hold implications for post-MI injection strategies (Rodell et al., 2013). A recent study has identified that targeted MI injections of a hyaluronic-based hydrogel, which eluted a recombinant MMP inhibitor, attenuated post-MI remodeling over and above that of hydrogel injections alone (Eckhouse et al., 2014). Since the present study identified that CHAM injections into the MI region modified MMP/cytokine expression profiles, then studies examining how this microsphere formulation may be synergistic with the addition of MMP/cytokine inhibitory molecules would be a potential future direction.

The clinical implications of these findings are 2-fold. First, changes in circulating MMP levels and macrophage phenotype have been shown to hold prognostic relevance in terms of post-MI remodeling in patients (Webb et al., 2006; Hohensinner et al., 2010). Thus, the effects of CHAM on these indices of MMP expression and macrophages identified in the present study are likely to be translatable and relevant. Second, the present study has identified a cellular basis by which biomaterials can modify the post-MI remodeling process, which included early transdifferentiation of myofibroblasts. Since the injection

of alginate-based biomaterial has advanced to clinical feasibility studies (LoneStar Heart, <http://clinicaltrials.gov/ct2/show/NCT01311791>), evaluating these cell phenotype changes would be a potentially important response variable.

Acknowledgments

The authors thank Ashley A. Sapp for editorial assistance.

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References

- Ahn MS (2007) Calcium hydroxylapatite: Radiesse. *Facial Plast Surg Clin North Am* 15:85–90, vii.
- Anzai A, Anzai T, Nagai S, Maekawa Y, Naito K, Kaneko H, Sugano Y, Takahashi T, Abe H, Mochizuki S, et al. (2012) Regulatory role of dendritic cells in postinfarction healing and left ventricular remodeling. *Circulation* 125:1234–1245.
- Baum J and Duffy HS (2011) Fibroblasts and myofibroblasts: what are we talking about? *J Cardiovasc Pharmacol* 57:376–379.
- Brown BN, Valentin JE, Stewart-Akers AM, McCabe GP, and Badylak SF (2009) Macrophage phenotype and remodeling outcomes in response to biologic scaffolds with and without a cellular component. *Biomaterials* 30:1482–1491.
- Burdick JA, Mauck RL, Gorman JH, 3rd, and Gorman RC (2013) Acellular biomaterials: an evolving alternative to cell-based therapies. *Sci Transl Med* 5:176–186.
- Chapman RE, Scott AA, Deschamps AM, Lowry AS, Stroud RE, Ikonomidis JS, and Spinale FG (2003) Matrix metalloproteinase abundance in human myocardial fibroblasts: effects of sustained pharmacologic matrix metalloproteinase inhibition. *J Mol Cell Cardiol* 35:539–548.
- Cohen HB and Mosser DM (2014) Cardiac macrophages: how to mend a broken heart. *Immunity* 40:3–5.
- Dewald O, Zymek P, Winkelmann K, Koerting A, Ren G, Abou-Khamis T, Michael LH, Rollins BJ, Entman ML, and Frangogiannis NG (2005) CCL2/Monocyte Chemoattractant Protein-1 regulates inflammatory responses critical to healing myocardial infarcts. *Circ Res* 96:881–889.
- Dixon JA, Gaillard WF, 2nd, Rivers WT, Koval CN, Stroud RE, Mukherjee R, and Spinale FG (2010) Heterogeneity in MT1-MMP activity with ischemia-reperfusion and previous myocardial infarction: relation to regional myocardial function. *Am J Physiol Heart Circ Physiol* 299:H1947–H1958.
- Dixon JA, Gorman RC, Stroud RE, Mukherjee R, Meyer EC, Baker NL, Morita M, Hamamoto H, Ryan LP, Gorman JH, 3rd, et al. (2011) Targeted regional injection of biocomposite microspheres alters post-myocardial infarction remodeling and matrix proteolytic pathways. *Circulation* 124(11, Suppl):S35–S45.
- Eckhouse SR, Purcell BP, McGarvey JR, Lobb D, Logdon CB, Doviak H, O'Neill JW, Shuman JA, Novack CP, Zellars KN, et al. (2014) Local hydrogel release of recombinant TIMP-3 attenuates adverse left ventricular remodeling after experimental myocardial infarction. *Sci Transl Med* 6:223–233.
- Epelman S, Lavine KJ, Beaudin AE, Sojka DK, Carrero JA, Calderon B, Brija T, Gautier EL, Ivanov S, Satpathy AT, et al. (2014) Embryonic and adult-derived resident cardiac macrophages are maintained through distinct mechanisms at steady state and during inflammation. *Immunity* 40:91–104.
- Ertl G and Frantz S (2005) Healing after myocardial infarction. *Cardiovasc Res* 66:22–32.
- Frangogiannis NG (2012) Regulation of the inflammatory response in cardiac repair. *Circ Res* 110:159–173.
- Freytes DO, Kang JW, Marcos-Campos I, and Vunjak-Novakovic G (2013) Macrophages modulate the viability and growth of human mesenchymal stem cells. *J Cell Biochem* 114:220–229.
- Goldsmith EC, Bradshaw AD, and Spinale FG (2013) Cellular mechanisms of tissue fibrosis. 2. Contributory pathways leading to myocardial fibrosis: moving beyond collagen expression. *Am J Physiol Cell Physiol* 304:C393–C402.
- Hohensinner PJ, Rychli K, Zorn G, Hülsmann M, Berger R, Mörtl D, Richter B, Huber K, Wojta J, Pacher R, et al. (2010) Macrophage-modulating cytokines predict adverse outcome in heart failure. *Thromb Haemostasis* 103:435–441.
- Ifkovits JL, Tous E, Minakawa M, Morita M, Robb JD, Koomalsingh KJ, Gorman JH, 3rd, Gorman RC, and Burdick JA (2010) Injectable hydrogel properties influence infarct expansion and extent of postinfarction left ventricular remodeling in an ovine model. *Proc Natl Acad Sci USA* 107:11507–11512.
- Ismahil MA, Hamid T, Bansal SS, Patel B, Kingery JR, and Prabhu SD (2014) Remodeling of the mononuclear phagocyte network underlies chronic inflammation and disease progression in heart failure: critical importance of the cardiosplenic axis. *Circ Res* 114:266–282.
- Jacovella PF (2008) Use of calcium hydroxylapatite (Radiesse) for facial augmentation. *Clin Interv Aging* 3:161–174.

- Johnson TD and Christman KL (2013) Injectable hydrogel therapies and their delivery strategies for treating myocardial infarction. *Expert Opin Drug Deliv* **10**: 59–72.
- Konstam MA, Kramer DG, Patel AR, Maron MS, and Udelson JE (2011) Left ventricular remodeling in heart failure: current concepts in clinical significance and assessment. *JACC Cardiovasc Imaging* **4**:98–108.
- Lambert JM, Lopez EF, and Lindsey ML (2008) Macrophage roles following myocardial infarction. *Int J Cardiol* **130**:147–158.
- Landa N, Miller L, Feinberg MS, Holbova R, Shachar M, Freeman I, Cohen S, and Leor J (2008) Effect of injectable alginate implant on cardiac remodeling and function after recent and old infarcts in rat. *Circulation* **117**:1388–1396.
- Lindsey ML, Goshorn DK, Squires CE, Escobar GP, Hendrick JW, Mingoa JT, Sweterlitsch SE, and Spinale FG (2005) Age-dependent changes in myocardial matrix metalloproteinase/tissue inhibitor of metalloproteinase profiles and fibroblast function. *Cardiovasc Res* **66**:410–419.
- Lindsey M, Wedin K, Brown MD, Keller C, Evans AJ, Smolen J, Burns AR, Rossen RD, Michael L, and Entman M (2001) Matrix-dependent mechanism of neutrophil-mediated release and activation of matrix metalloproteinase 9 in myocardial ischemia/reperfusion. *Circulation* **103**:2181–2187.
- Ma Y, Halade GV, Zhang J, Ramirez TA, Levin D, Voorhees A, Jin YF, Han HC, Manicone AM, and Lindsey ML (2013) Matrix metalloproteinase-28 deletion exacerbates cardiac dysfunction and rupture after myocardial infarction in mice by inhibiting M2 macrophage activation. *Circ Res* **112**:675–688.
- Morita M, Eckert CE, Matsuzaki K, Noma M, Ryan LP, Burdick JA, Jackson BM, Gorman JH, 3rd, Sacks MS, and Gorman RC (2011) Modification of infarct material properties limits adverse ventricular remodeling. *Ann Thorac Surg* **92**: 617–624.
- Mukherjee R, Zavadzka JA, Saunders SM, McLean JE, Jeffords LB, Beck C, Stroud RE, Leone AM, Koval CN, Rivers WT, et al. (2008) Targeted myocardial micro-injections of a biocomposite material reduces infarct expansion in pigs. *Ann Thorac Surg* **86**:1268–1276.
- Nahrendorf M and Swirski FK (2013) Monocyte and macrophage heterogeneity in the heart. *Circ Res* **112**:1624–1633.
- Pilla JJ, Gorman JH, 3rd, and Gorman RC (2009) Theoretic impact of infarct compliance on left ventricular function. *Ann Thorac Surg* **87**:803–810.
- Rane AA, Chuang JS, Shah A, Hu DP, Dalton ND, Gu Y, Peterson KL, Omens JH, and Christman KL (2011) Increased infarct wall thickness by a bio-inert material is insufficient to prevent negative left ventricular remodeling after myocardial infarction. *PLoS ONE* **6**:e21571.
- Rodell CB, Kaminski AL, and Burdick JA (2013) Rational design of network properties in guest-host assembled and shear-thinning hyaluronic acid hydrogels. *Biomacromolecules* **14**:4125–4134.
- Shumaker PR, England LJ, Dover JS, Ross EV, Harford R, Derienzo D, Bogle M, Uebelhoefer N, Jacoby M, and Pope K (2006) Effect of monopolar radiofrequency treatment over soft-tissue fillers in an animal model: part 2. *Lasers Surg Med* **38**: 211–217.
- Shuman JA, Zurcher JR, Sapp AA, Burdick JA, Gorman RC, Gorman JH, 3rd, Goldsmith EC, and Spinale FG (2013) Localized targeting of biomaterials following myocardial infarction: a foundation to build on. *Trends Cardiovasc Med* **23**:301–311.
- Spinale FG (2007) Myocardial matrix remodeling and the matrix metalloproteinases: influence on cardiac form and function. *Physiol Rev* **87**:1285–1342.
- Spinale FG, Janicki JS, and Zile MR (2013) Membrane-associated matrix proteolysis and heart failure. *Circ Res* **112**:195–208.
- Tomasek JJ, Gabbiani G, Hinz B, Chaponnier C, and Brown RA (2002) Myofibroblasts and mechano-regulation of connective tissue remodelling. *Nat Rev Mol Cell Biol* **3**:349–363.
- Tous E, Ifkovits JL, Koomalsingh KJ, Shuto T, Soeda T, Kondo N, Gorman JH, 3rd, Gorman RC, and Burdick JA (2011) Influence of injectable hyaluronic acid hydrogel degradation behavior on infarction-induced ventricular remodeling. *Biomacromolecules* **12**:4127–4135.
- Tous E, Weber HM, Lee MH, Koomalsingh KJ, Shuto T, Kondo N, Gorman JH, 3rd, Lee D, Gorman RC, and Burdick JA (2012) Tunable hydrogel-microsphere composites that modulate local inflammation and collagen bulking. *Acta Biomater* **8**: 3218–3227.
- van der Laan AM, Ter Horst EN, Delewi R, Begieneman MP, Krijnen PA, Hirsch A, Lavaei M, Nahrendorf M, Horrevoets AJ, Niessen HW, et al. (2014) Monocyte subset accumulation in the human heart following acute myocardial infarction and the role of the spleen as monocyte reservoir. *Eur Heart J* **35**:376–385.
- Webb CS, Bonnema DD, Ahmed SH, Leonardi AH, McClure CD, Clark LL, Stroud RE, Corn WC, Finklea L, Zile MR, et al. (2006) Specific temporal profile of matrix metalloproteinase release occurs in patients after myocardial infarction: relation to left ventricular remodeling. *Circulation* **114**:1020–1027.
- Widgerow AD (2011) Cellular/extracellular matrix cross-talk in scar evolution and control. *Wound Repair Regen* **19**:117–133.
- Yushkevich PA, Piven J, Hazlett HC, Smith RG, Ho S, Gee JC, and Gerig G (2006) User-guided 3D active contour segmentation of anatomical structures: significantly improved efficiency and reliability. *Neuroimage* **31**:1116–1128.

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