Targeted Injection of a Biocomposite Material Alters Macrophage and Fibroblast Phenotype and Function Following Myocardial Infarction: Relation to LV Remodeling

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Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
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<tr>
<td>CCR2</td>
<td>C-C chemokine receptor type 2</td>
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<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
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<td>CHAM</td>
<td>Calcium hydroxyapatite microspheres</td>
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<td>COL1a1</td>
<td>Collagen type-1</td>
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<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
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<tr>
<td>EDV</td>
<td>End-diastolic volume</td>
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<td>EF</td>
<td>Ejection fraction</td>
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<td>IL</td>
<td>Interleukin</td>
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<td>LGE</td>
<td>Late-gadolinium enhanced</td>
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<td>LTBP</td>
<td>Latency binding protein</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<td>LV</td>
<td>Left ventricular</td>
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<td>MCP</td>
<td>Monocyte chemotactic protein</td>
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<td>MI</td>
<td>Myocardial infarction</td>
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<td>MIP</td>
<td>Macrophage inflammatory protein</td>
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<td>MMP</td>
<td>Matrix metalloproteinase</td>
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<td>mRNA</td>
<td>Messenger RNA</td>
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<td>PCR</td>
<td>Polymerase chain reaction</td>
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<td>PSR</td>
<td>Picro-sirius red</td>
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<td>RNA</td>
<td>Ribonucleic acid</td>
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<td>SMA</td>
<td>Smooth muscle actin</td>
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<td>SSFP</td>
<td>Steady State Free Precession</td>
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<td>TGF</td>
<td>Transforming growth factor beta</td>
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<td>TNF</td>
<td>Tumor necrosis factor</td>
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Abstract

A treatment target for progressive 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 (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 (MAC) and fibroblast (FIBRO), were examined. MI was induced in adult pigs before randomization to CHAM injections (20, 0.1mL, targeted injections within MI region) or saline. At 7 or 21 days post-MI (n=6/time point/group), cardiac MRI was performed, followed by MAC and FIBRO isolation. Isolated MAC profiles for monocyte chemotactic MAC inflammatory protein-1 (MCP-1) as measured by rtPCR increased at 7 days post-MI in the CHAM group compared to MI only (16.3±6.6 vs 1.7±0.6 Ct values, p<0.05) and were similar by 21 days post-MI. Temporal changes in FIBRO function and smooth muscle actin (SMA) expression relative to referent control (n=5) occurred with MI. CHAM induced increases in FIBRO proliferation, migration, and SMA expression - indicative of FIBRO transformation. By 21 days, CHAM reduced LV dilation (diastolic volume: 75±2 vs 97±4 mL) and increased function (ejection fraction: 48±2 vs 38±2 %) compared to 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 biomaterial injections impart biological 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 is characterized by progressive extension and thinning of the MI region, termed infarct expansion. (Konstam et al., 2011; Ertl and Frantz, 2005; Spinale, 2007; 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. While 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. (Frangogiannis, 2012; Hohensinner et al., 2010; Dewald et al., 2005) 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. (Morita et al., 2011; Tous et al., 2012; Mukherjee et al., 2008; Ifkovits et al., 2010; Pilla et al., 2009; Rane et al., 2011; Shuman et al., 2013) One of the prototype biomaterials that has been evaluated in pre-clinical post-MI models is those based upon a hydroxyapatite composition. (Morita et al., 2011; Mukherjee et al., 2008; 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. (Morita et al., 2011; Mukherjee et al., 2008; Shuman et al., 2013; Dixon et al., 2011) Secondly, 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. (Morita et al., 2011; Dixon et al., 2011) While 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. (Frangogiannis, 2012; Hohensinner
et al., 2010; Dewald et al., 2005; Baum and Duffy, 2011; Tomasek et al., 2002; Goldsmith et al., 2013; Widgerow, 2011; Lindsey et al., 2005; Chapman et al., 2003) Specifically, a shift in the expression profile of macrophages, termed macrophage polarization, occurs in the post-MI period which can affect post-MI remodeling. (Frangogiannis, 2012; Brown et al., 2009; Anzai et al., 2012; Ma et al., 2013) The proliferation of fibroblasts with a more smooth muscle like phenotype occur within the MI region, and this process has been termed fibroblast transdifferentiation, and these fibroblasts termed myofibroblasts. (Spinale, 2007; Tomasek et al., 2002; 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 utilized whereby injections of calcium hydroxyapatite microspheres (CHAM; carboxymethylcellulose, Radiesse™, Bioform Medical Inc., San Mateo, CA) were performed at the time of MI induction. (Morita et al., 2011; Dixon et al., 2011) CHAM has been used clinically as dermal fillers for facial augmentation whereby the CHAM microspheres (25-45 micron diameter) are suspended in a gel carrier composed of glycerin (Jacovella, 2008; Ahn, 2007). The disposition of CHAM following injections is that of a bioerodible, whereby the gel carrier degrades quickly and is then followed by more prolonged cell dependent biodegradable process of the microspheres. (Jacovella, 2008; Ahn, 2007; Dixon et al., 2011; Shuman et al., 2013; Tous et al., 2012) More specifically, CHAM is cleared by a macrophage dependent process, which is then accompanied by proliferation of fibroblasts. (Jacovella, 2008; Ahn, 2007; Shumaker et al., 2006; 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. (Ertl and Frantz, 2005; Frangogiannis, 2012; Freytes et al., 2013; Goldsmith et al., 2013; Hohensinner et al., 2010; Lindsey et al., 2001) Past studies have provided functional evidence that the effects of injected acellulular biomaterials, such as CHAM upon LV geometry and post-MI remodeling, is 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; Shuman et al., 2013; Tous et al., 2012; Tous et al., 2011; Burdick 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. (Konstam et al., 2011; Ertl and Frantz, 2005;
Spinale, 2007; Frangogiannis, 2012) Accordingly, LV geometry and function, indices of ECM remodeling, as well as studies of isolated of macrophages and fibroblasts, were performed at these post-MI time points in order 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 this model was utilized in the present study. (Frangogiannis, 2012; Dixon et al., 2010) Briefly, pigs (n=24, Yorkshire, 25kg) 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. (Frangogiannis, 2012; Dixon et al., 2010) Platinum markers (10, 2 mm) were sutured around the periphery of the MI region in order 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 was performed. Each injection was performed using a 27 gauge needle placed into the mid-myocardial wall whereby 150 uL 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 semi-solid 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/time points). 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 3D SSFP cine MRI for volumetric analysis using the following parameters: field of view – 300 x 244, acquisition matrix – 192 x 156, repetition time – 3.11ms, echo time – 1.53ms, bandwidth – 1184Hz/pixel, slice thickness – 4mm, averages – 2. Fifteen minutes following intravenous injection of 0.1mmol/kg gadobenate dimeglumine (MultiHance; Bracco Diagnostics, Princeton, NJ), infarcts were visualized using a 3D late-gadolinium enhanced (LGE) spoiled gradient echo sequence with the following parameters: field of view – 350 x 350, acquisition matrix – 256 x 256, repetition time – 591.28ms, echo time – 2.96ms, inversion time – 200-300ms, flip angle - 25˚, averages – 2. Imaging data sets were blinded to the end-user throughout post-processing. LV volume and global function data was obtained using prospective SSFP cine MRI images. (Yushkevich et al., 2006) Raw short-axis images were automatically sorted, cropped, and contrast normalized in a custom Matlab (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 semi-automated 3D active contour segmentation program (ITK-SNAP, open access/source). LV end-diastolic volume (EDV) and ejection fraction (EF) 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, 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., 2011; Dixon et al., 2010) For RNA extraction, MI samples were placed in an initial RNA extraction solution (RNA Later, Life Technologies) and then fully extracted (RNeasy Fibrous Tissue Mini Kit, Qiagen), whereby the quantity and quality of the RNA was determined (Experion Automated Electrophoresis System; Bio-Rad Laboratories, Hercules, CA). RNA (1 ug) was reverse transcribed to generate cDNA (iScript cDNA Synthesis Kit; Bio-Rad, Hercules, CA). 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 (COL1a1), transforming growth factor beta (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, Hercules, CA). The real time PCR fluorescence signal was converted to cycle times (Ct) normalized to GAPDH (ΔCt) and final results expressed as $2^{\Delta C_{\text{t}}} \times 10^3$. All PCR assays were performed in duplicate.

The formalin fixed full thickness MI samples were embedded, sectioned (7 um), and stained with picrosirius red for fibrillar collagen, and the percent area of collagen within the remote and MI regions were computed using computer assisted morphometry. (Mukherjee et al., 2008; Dixon et al., 2010)
2011) Immunostaining was used to colocalize with cells that stained positive for α-smooth muscle actin (1:100; α-smooth muscle actin: Sigma A5228), a marker for actin present in vascular smooth muscle cells as well as myofibroblasts, using approaches described previously. (Dixon et al., 2011; Lindsey et al., 2005)

Macrophage Isolation

Macrophages were isolated from the MI region using methods detailed previously. (Ma et al., 2013; Brown et al., 2009; Freytes et al., 2013) Briefly, the MI samples (500 mg) were minced and incubated in a digestion solution (Liberase Blendzyme, 0.25 mg/mL:37°C, Roche#540189001) with gentle trituration for 60 min, resuspended in cold buffer (PEB: BSA, PBS, autoMACS rinsing solution (Miltenyl Biotec, Cat #130-091-222)), and centrifuged (300g,10min) with these last steps repeated (X3). The suspension was filtered (Miltenyi Biotec, Cat #130-041-407) and cell density determined (hemocytometry), diluted to 2X10^8 cells/mL, and the cell suspension incubated with CD11b microbeads (Miltenyi Biotec, Cat #130-049-601) and run over a magnetic separation column (Mini MACSTM separator) and rinsed with PEB. The retained CD11b cells (macrophages) were eluted into culture media (DMEM), placed into aliquots at a final concentration of 2X10^7/100 uL. RNA was isolated from these macrophages as described in the previous paragraph, and PCR performed for cytokine expression of porcine specific tumor necrosis factor alpha (TNF), interleukin-8 (IL-8), monocyte chemotactic protein-1 (MCP-1), and macrophage inflammatory protein-1 alpha (MIP-1A).

Fibroblast Isolation

Fibroblasts from the MI region were isolated using selective collagenase digestion as described previously. (Lindsey et al., 2005; Chapman et al., 2003) Fibroblasts were maintained in tissue culture flasks (150 cm^2; Falcon, Fisher Scientific, Pittsburgh, PA) with complete growth media
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(Fibroblast Growth Medium, Promocell, Catalog #C23010, Heidelberg, Germany) containing 17% fetal bovine serum (Catalog #10082-147, Invitrogen, Carlsbad, CA) and Promocell Fibroblast Growth Supplement (Catalog #C39315). 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 Bioscience, CA) and incubated for 24 hours. Fibroblast migration into the bottom chamber was determined by fluorescence (CyQuant).

Data Analysis

The treatment assignments were pre-determined and each animal 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 (STATA Corp, 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 a mean ±
standard error of the mean (SEM), 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 an MI only (saline injection) and MI/CHAM are shown in Figure 1. LV dilation and MI wall thinning was evident in both MI groups but appeared to be attenuated with CHAM injection. LV EDV was increased and LV EF reduced when compared to 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 (Figure 1). Thus, consistent with past reports, targeted CHAM injection within the MI region favorably altered the course of adverse LV remodeling post-MI. (Morita et al., 2011; Dixon 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 (Figure 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 PSR staining, a clear fibrotic pattern was observed in all post-MI groups (Figure 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 SMA staining was observed in fibroblasts from both MI groups (Figure 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 (Figure 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 to the MI only group at 7 days post-MI (Figure 5).


Discussion.

Strategies to modify stress/strain patterns post-MI utilizing mechanical restraint have been performed at both the global and regional level. (Morita et al., 2011; Tous et al., 2012; Mukherjee et al., 2008; Ifkovits et al., 2010; Pilla et al., 2009; Rane et al., 2011; Shuman et al., 2013) Specifically, a number of past studies using either a hydroxyapatite based or alginate based biomaterial have demonstrated favorable effects on LV geometry and function post-MI. (Morita et al., 2011; Tous et al., 2012; Mukherjee et al., 2008; Shuman et al., 2013; Dixon et al., 2011) 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 two-fold. First, in the early post-MI period (7 days), CHAM injections reduced mRNA levels for MMP types, which have been implicated to contribute 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 for the first time demonstrated 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. (Spinale, 2007; Morita et al., 2011; Tous et al., 2012; Frangogiannis, 2012; Hohensinner et al., 2010; Dewald et al., 2005) The second phase is heralded by proliferation and transdifferentiation of myocardial fibroblasts into myofibroblasts. (Baum and Duffy, 2011; Tomasek et al., 2002; Goldsmith et al., 2013; Widgerow, 2011) Further, induction of ECM structural proteins, such as fibrillar collagens, mediated in part by profibrotic signaling molecules such as TGF, occur. 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) While 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, 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 which have been implicated to cause adverse LV remodeling post-MI.\(^2\) 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.\(^2\) Thus, the reduction in these specific MMP types with CHAM injections likely reduced overall ECM proteolysis. One of the major sources of MMP-9 is that of neutrophils, and thus the early induction of MMP-9 in both humans and animals following MI is likely from this inflammatory cell type.\(^3\) Indeed, the acute magnitude of MMP-9 release has been associated with worsening post-MI remodeling in patients.\(^3\) While 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.\(^4\) In the present study, isolated macrophage expression profiles indicated a greater shift in the M1 phase, as indicated by increased TNF and IL-8.\(^4\) Moreover, CHAM injections induced key factors in macrophage function, such as MCP-1 and MIP-1A, which can directly influence post-MI remodeling.\(^5\) 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 to 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; Frangogiannis, 2012; Nahrendorg and Swirski, 2013; Lambert et al., 2008) 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 over-simplistic. There appears to be several macrophage subtypes within the myocardium, and consequently a much greater heterogeneity of macrophages exist, thus this polarization classification may be too general (Frangogiannis, 2012; Nahrendorg and Swirski, 2013; Epelman et al., 2014; Cohen and Mosser, 2014) For example, using genetic fate mapping and angiotensin-II infusions in mice, it was demonstrated that unique sub-populations of resident macrophages exist within the myocardium, notably the MCP-1 receptor (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 an over 3-fold reduction in MMP-9 expression, predominantly expressed in activated lymphocytes. While associative, these findings would suggest that a shift in the subpopulations of macrophages resident within the MI was induced by CHAM injections. Taking these observations and the recent findings that a diverse set of myocardial macrophage subpopulations exist and influence local response to
myocardial stress, (Epelman et al., 2014; Cohen and Mosser, 2014) then future studies that more carefully phenotype these macrophage subpopulations with MI and CHAM injections would be warranted. In addition, while 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. (van der Laan et al., 2014; Ismahil 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 upon macrophages isolated strictly from the MI region in order to focus upon 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, was affected by CHAM injections remains to be determined.

Myocardial fibroblast phenotype post-MI; Effects of CHAM injection

While 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. (Baum and Duffy, 2011; Tomasek et al., 2002; Goldsmith et al., 2013; Widgerow, 2011) In addition, the 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 (Dixon et al., 2011; Baum and Duffy, 2011; Lindsey et al., 2005) and was utilized 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. (Goldsmith et al., 2013; Widgerow, 2011; Lindsey et al., 2005) 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 pro-fibrotic 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 only have reduced ECM proteolysis but also affected the profibrotic response. Indeed, histological 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

While 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, reduce adverse post-MI remodeling, and as such, translational studies regarding the underlying mechanisms and pathways by which these materials are operative has
accelerated. (Morita et al., 2011; Tous et al., 2012; Mukherjee et al., 2008; Ifkovits et al., 2010; Pilla et al., 2009, Rane et al., 2011; Shuman et al., 2013; Tous et al., 2012; Tous et al., 2011; Burdick et al., 2013; Landa et al., 2008; Johnson and Christman, 2013) Using myocardial injections of a hyaluronic acid and poly(lactide-co-glycolide) based microsphere (~50 microns) 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., 2012; Tous et al., 2011) 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 similar microsphere size and concentration (Jacovella, 2008; Ahn, 2007) to this past study, has also been shown to reduce LV dilation in an ovine model for up to 8 weeks post-injection. (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 the past evidence in large animal models that injections of this microsphere formulation was 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. (Jacovella, 2008; Ahn, 2007) 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 biological 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 to non-injected 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 utilized an intra-operative myocardial injection method to deliver the biomaterial to the MI region. (Dixon et al., 2011; Morita et al., 2011; Mukherjee et al., 2008; Pilla et al., 2009; Rane et al., 2011; Tous et al., 2012; Landa et al., 2008) While 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. (Shuman et al., 2013; Burdick et al., 2013; Johnson 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. (Hohensinner et al., 2010; Webb et al., 2006) 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, 2011) evaluating these cell phenotype changes would be a potentially important response variable.
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Authorship Contributions

Participated in research design: Burdick, Gorman JH, Gorman RC, Spinale

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References


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**Figure Legends.**

**Figure 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 an MI only and an 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 EDV was increased in both MI groups compared to referent normal values (54±6 mL, p<0.05) at 7 days with no difference between groups. However, by 21 days post-MI, LV EDV had increased further in the MI only group but was attenuated in the MI/CHAM group. LV EF was reduced in both MI groups at 7 days post-MI from referent control (56±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 vs respective MI only values)

**Figure 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 to MI only, and MMP-9 mRNA levels remained reduced from MI only values at 21 days post-MI. Collagen type-1 and LTBP mRNA levels were lower in the MI CHAM group at 7 days post-MI. All results are expressed as ΔCt X 10^3. (Sample sizes - MI only: n=6/time point; MI/CHAM: n=6/time point; referent normal controls: n=5; *p<0.05 vs referent control, +p<0.05 vs respective MI only, #p<0.05 vs Week 1 values)

**Figure 3.** Histological staining for fibrillar collagen with 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 to MI only values. Original image magnification 40X. (Sample sizes - MI only: n=6/time point; MI/CHAM: n=6/time point; referent normal controls: n=5; *p<0.05 vs referent control, +p<0.05 vs respective MI only values)

**Figure 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 vs respective MI only, #p<0.05 vs Week 1 values)

**Figure 5.** Fibroblasts isolated from the MI region were analyzed with respect to proliferation and migration rates and compared to 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 vs referent control, +p<0.05 vs respective MI only, #p<0.05 vs Week 1 values)
Figure 1.
Figure 2.
Figure 3.

The figure shows immunohistochemical staining of tissues from different groups: Control, 7-Days MI Only, 7-Days CHAM, 21-Days MI Only, and 21-Days CHAM. The images are labeled for PSR and SMA. The bar graphs on the right show the percentage of positive staining (PSR and SMA) for each group at 7-Days and 21-Days post MI. Statistically significant differences are marked with asterisks.
Figure 4.
Figure 5.

Fibroblast Proliferation

Fibroblast Migration

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