Enhanced intramyocardial vascular cell delivery promotes post-myocardial infarction healing by polarizing pro-regenerative neutrophils

Authors and Affiliations

Xuechong Hong [1,2], Allen Chilun Luo [1], Ilias Doulamis [1,2], Nicholas Oh [1,2], Pedro J. del Nido [1,2], Juan M. Melero-Martin [1,2,3], Ruei-Zeng Lin [1,2]

[1] Department of Cardiac Surgery, Boston Children's Hospital, Boston, MA 02115, USA
[2] Department of Surgery, Harvard Medical School, Boston, MA 02115, USA
[3] Harvard Stem Cell Institute, Cambridge, MA 02138, USA

Corresponding author

Ruei-Zeng Lin, Ph.D.
Department of Cardiac Surgery
Boston Children's Hospital
Harvard Medical School
300 Longwood Ave., Enders 345
Boston, MA 02115
Tel.: (617) 919-1905
Fax: (617) 730-0235
E-mail: ruei-zeng.lin@childrens.harvard.edu

Abstract

Success of vascular progenitor cell transplantation for cardiac regeneration is partially limited by low retention/engraftment of the delivered cells. A clinically applicable method for improved cell retention would enable optimization of cell delivery. In this study, we used the myocardial infarction (MI) model by ligating the left anterior descending arteries in mice. We developed a new intramyocardial cell delivery technique using photocrosslinkable GelMA hydrogel to significantly enhance the therapeutic cell engraftment in beating hearts. Based on this technical breakthrough, we reported for the first time that the mechanism of vascular cell delivery promotes post-myocardial infarction healing by polarizing pro-regenerative neutrophils. TGFβ produced by endothelial cells engrafted with proper perivascular interaction polarized the recruited bone marrow neutrophils toward pro-regenerative phenotype. Depletion host neutrophils by specific antibody treatment canceled the therapeutic benefits of co-delivering of human ECFCs and MSCs in ischemic hearts, indicating the non-inflammatory, pro-regenerative neutrophils are indispensable mediators of beneficial cardiac remodeling.

Introduction

Myocardial infarction (MI) remains the leading cause of morbidity and mortality worldwide [1,2]. An MI results in irreversible damage to the heart muscle due to a blockage of coronary arteries. Myocardial necrosis starts rapidly after the onset of ischemia, leaving an infarct zone that contains nonfunctional myocytes and excessive inflammatory cells [3]. The loss of viable myocardium initiates a process of adverse ventricular remodeling and the formation of fibrotic scar tissue. MI patients suffer from the decreased cardiac output, resulting in a downward spiral leading to congestive heart failure. In clinical, acute mortality rate of MI has fallen dramatically through current surgical interventions aided by pharmacotherapy and mechanical devices. Notwithstanding the short-term benefit of these interventions to resolve the ischemia, only modest improvements in global cardiac functions six months after an acute MI were observed in patients

[4,5]. A significant number of patients go on to develop late sequelae, including heart failure and arrhythmia [2]. Heart muscle salvage after MI is the single important determinant factor for event-free long-term survival [2].

While the human heart is considered a terminally differentiated organ, adult cardiac regeneration by endogenous repair mechanisms is considered unfeasible with current medical options. Over the last decade, many investigators have tested cell-based therapies aimed at preventing adverse ventricular remodeling and promoting recovery of MI in animal models, as well as in clinical trials [6–8]. Delivery of therapeutic cells to the heart is performed in two main categories. Cardiac patches or cell sheets containing human iPSC-derived cardiomyocytes are transplanted to reinforce or replace the infarct myocardium aiming to rebuild the loss of heart walls. These constructs were mainly manufactured by seeding cells in biomaterial scaffolds and preconditioned in a bioreactor before surgical implantation. Other approaches inject therapeutic cells directly into the infarcted areas to mitigate the detrimental impact of myocardial infarction. Delivery of cells by intramyocardial or intracoronary injections potentially allows the early therapeutic intervention in the acute MI phase. Examples of adult stem/progenitor cells that have been tested are cardiosphere-derived cells, bone marrow-derived mononuclear cells, endothelial progenitor cells, and mesenchymal stem cells. Depending on the cell types, the long-term engraftment or transdifferentiation into myocytes of injected cells was shown to be unnecessary to exhibit the therapeutic effect. Accumulating evidence suggested this beneficial effect through paracrine factors aiding cardiac remodeling or the modulation of inflammatory response. However, the underlying mechanisms are not well-understood.

Delivering vascular progenitor cells is a promising new treatment modality for ischemic cardiomyopathy. Some studies have focused on the delivery of vascular cells to build new blood vessels and/or stimulate angiogenesis, based on the concept that rapid restoration of microvascular networks within the ischemic myocardium would mitigate adverse remodeling. However, low cell retention and engraftment are major obstacles to achieving a significant functional benefit. In a rat myocardial ischemia/reperfusion injury (IRI) model, animals injected with human endothelial colony forming cells (ECFCs) and mesenchymal stem cells (MSCs) in PBS revealed modest reduction in adverse ventricular remodeling. Quantitative analysis shown a rapid loss of human cells shortly after myocardial injection, suggesting the lack of sufficient cellular retention could be the major reason of weak effect. Therefore, it is still an open question whether human vascular progenitor cells with vasculogenic capability can mitigate adverse remodeling and promote functional recovery.

In this study, our approach is to use well-characterized Gelatin methacryloyl (GelMA) hydrogel to deliver human endothelial colony-forming cells (ECFCs) and mesenchymal stem cells (MSCs) to build neo-vascular networks in ischemic myocardium. GelMA was synthesized in our previous studies to be a photocrosslinkable, bioadhesive hydrogel following its injection in tissues. GelMA formulation can polymerize rapidly (10 seconds upon exposure to UV light in the presence of a photoinitiator). We proposed that this rapid polymerization would be a critical feature to avoid hydrogel dissemination at the implantation site. Recently, we also demonstrated that this material is fully compatible with ECFC-based vascular morphogenesis, and thus we proposed its use for vascular cell therapy in infarct hearts. Here, we demonstrated that GelMA hydrogel can be transmyocardially photocrosslinked and maintain vascular cell retention in infarcted mouse hearts. GelMA-enabled cell delivery promoted infarct healing and adaptive remodeling to preserve cardiac function. Interestingly, enhanced cell retention allows us to study and discover for the first time the early beneficial effect of human vascular cells in MI. We found that the enhanced retention of human ECFCs and MSCs enabled the perivascular cell-cell interactions to upregulate TGFb expression. TGFb signaling promoted the polarization of non-inflammatory, pro-regenerative neutrophils, which are indispensable mediators of beneficial cardiac remodeling.

Results

In situ polymerization of GelMA hydrogel in infarcted myocardium

Transmyocardial polymerization of GelMA was evaluated in vivo using a mouse MI model. MI was induced by permanent ligation of the left anterior descending (LAD) arteries (**Figure S1** and **Video S1**, Supporting Information). A mixture of human vascular progenitor cells (ECFCs and MSCs; $5x10^5$ for each cell type) suspended in 1% (w/v in PBS) GelMA precursor solution was injected into the infarct myocardium within 10 min of LAD ligation. The total injection volume per heart is 100 µL. We first demonstrated that UV light can transmit through the myocardium of mice using our OmniCure S2000 UV lamp (wavelength 320-500 nm; the intensity of 40 mW/cm^2). GelMA polymerization was achieved after just 10 seconds of transmyocardial exposure to UV light (**Video S2**, Supporting Information). The general safety of short UV illumination by our UV lamp had been evaluated in our previous study [9]. Our working range of UV light exposure (10 - 45 seconds) was deemed sufficiently safe for both injected cells and surrounding tissues [9]. Moreover, we did not observe leakage of GelMA solution or swelling of local tissues with our injection volume and crosslinking conditions.

GelMA hydrogel enhanced cell retention in ischemic hearts

To evaluate the acute myocardial retention of human vascular cells, we injected $5x10^5$ luciferase-labeled ECFCs (luc-ECFCs) with $5x10^5$ non-labeled MSCs per heart and quantified the bioluminescence intensity in the infarcted area (**Figure 1D**). We compared transmyocardial photocrosslinking of cell-laden GelMA following injection into infarcted hearts (referred to as GelMA-Hydrogel or **GelMA-H** groups; **Figure 1C**) with the results of cell injection in the same GelMA precursor solution but without UV illumination (GelMA-Solution or **GelMA-S** groups; **Figure 1B**). Notably, the GelMA precursor solution remained liquid in physiological conditions (i.e., at 37^oC and in contact with blood or tissues) if not crosslinked by UV. Since the cardiac contraction and vessel perfusion had been proved to be the primary culprits in the early washout of cells from the injection site[10], we injected the same amount of human cells into the arrested mouse hearts and performed bioluminescence measurement in 15 mins to obtain a baseline. Bioluminescence signals compared to this baseline represent an estimated percentage of ECFC retention at the given conditions (**Figure 1E**).

As early as 3h after MI induction and cell delivery, the ECFC retention in GelMA-S groups reduced to 20% (**Figure 1D-E**), a low retention rate in accordance with previous studies that quantified the efficiency of cell delivery in plain PBS [10–12]. The potential reason that cardiac contraction reduces cell retention is by active extrusion of the liquid injectate during each heartbeat. We tested this notion by in situ photopolymerization of GelMA hydrogel to minimize backwash. In the GelMA-H group, ECFC retention was significantly increased to 48% (3 h, $p < 0.01$ vs. cells in GelMA-S), revealing a dramatic effect of sealing the injection site on cell retention. The higher ECFC retention in the GelMA-H group was maintained throughout the 3-day monitoring post-MI (reduced 50% from 48% to 24% from 3h to 72h). In comparison, the injected cells in GelMA-S lost rapidly within the initial 24 hours (reduced 70% from 20% to 6% from 3h to 72h).

To confirm that the GelMA-H enhanced the retention of both endothelial and mesenchymal cells, we quantified non-labeled ECFCs and MSCs in infarcted hearts by flow cytometry (**Figure S2**, Supporting Information). Cell delivery in GelMA-H enhanced both ECFC and MSC retentions at 48 h compared to the GelMA-S group (**Figure 1F**). Notably, GelMA hydrogel maintained the engraftment of ECFCs and MSCs close to the original 1:1 ratio, which was optimized to achieve a long-term vascularization [13]. Collectively, our data suggest that the rapid in situ photopolymerization of GelMA hydrogel is adequate to protect human cells and reduce the "washout" effect during the initial phase (72h) of therapeutic cell engraftment.

Human vascular cells delivered in GelMA hydrogel promoted beneficial cardiac remodeling

To determine whether the increase in acute cell retention would impact MI healing, we used echocardiography to evaluate the cardiac function (**Figure 2A and S3**, Supporting Information). Echocardiography revealed no significant difference in baseline cardiac function between groups measured 1 h before LAD ligation. On day 7 following the induction of MI, left ventricular fractional shortening (LVFS) and left ventricular ejection fraction (LVEF), two measurements of global cardiac function, were higher in the mice treated with human cells delivered in GelMA-H compared to the untreated or GelMA-S-treated animals (**Figure 2B-C**). After treatment, both cardiac functions were significantly higher in the GelMA-H group. Compared with the GelMA-S group, LVEF and LVFS in the GelMA-H group were improved considerably in the measurements on days 7, 14, and 21. Both untreated or GelMA-S-treated groups showed a continuous decline in LVEF and LVFS from day 3 to 28 post-MI, indicating a deterioration of cardiac function due to adverse infarct remodeling. There is no significant difference in LVEF and LVFS between untreated or GelMA-S-treated groups, suggesting the lack of therapeutic benefit for low cell retention. In contrast, in the GelMA-H group, cardiac function was preserved from day 3 to day 28.

Heart muscle salvage after MI is the single important determinant factor for the long-term preservation of cardiac function and survival [2]. We measured the myocardial wall thickness by echocardiography. As shown in **Figure 2D**, the GelMA-H group showed a significantly higher left ventricular anterior wall thickness (LVAW) compared to the untreated or GelMA-S groups. Again, no improvement was found by comparing GelMA-S and untreated groups, suggesting that high cell retention was required for beneficial cardiac remodeling. The preservation of the myocardium was confirmed by histological analysis of cross-sections in the infarct area (**Figure 2E**). In animals that received cell delivery in GelMA-H, myocardial thickness significantly improved compared to the untreated or GelMA-S groups (**Figure 2F**). Immunofluorescent staining confirmed the higher survival of cardiomyocytes (cTnT+) within the infarct area of GelMA-H mice (**Figure 2G-H**). Fluorescent image assessment demonstrated that the number of capillaries in GelMA-H-treated hearts was significantly higher in both the border zone and infarcted area than in the control groups. Masson's trichrome staining of cardiac tissues harvested at 4 weeks also confirmed that the GelMA-H group showed significantly reduced myocardial fibrosis compared to the untreated or GelMA-S-treated groups (**Figure 2I-J**). Taken together, our data indicate that optimizing acute cell retention translates to a superior functional benefit, including better revascularization, less scar tissue formation, and preservation of overall cardiac functions after MI.

Functional human vasculature in infarct hearts

We sought to investigate the mechanism of therapeutic benefit conduced by GelMA-enhanced human vascular cell delivery. First, we examined the infarct hearts on day 7 to assess the vasculogenesis of human cells [14]. The histological examination carried out by immunofluorescence staining of human-specific vimentin and UEA1 revealed that the extent of vascular network formation differed in the GelMA-H and GelMA-S groups (**Figure 2K**). Significant engraftment of human ECFCs and MSCs in the GelMA-H groups was evidenced by the formation of human-specific vascular networks inside the infarct myocardium (**Figure 2L**). In the GelMA-S groups, only a few human MSCs, but not ECFCs, were stained in the infarct myocardium. Taking together, our data suggested the in situ photocrosslinking of GelMA hydrogel significantly improved the retention of co-delivered human ECFCs and MSCs. Without the timely gelation of GelMA hydrogel, although some MSCs could engraft, ECFCs failed to participate in the revascularization of infarcted myocardium.

The formation of functional human vasculature in ischemic tissues might explain parts of the therapeutic benefit observed in the GelMA-H group. We noticed that echocardiography showed improved cardiac functions on as early as day 7 (LVFS and LVEF; **Figure 2B-C**). Significant improvement of LVAW in the GelMA-H group (**Figure 2D**) was even observed on as early as day 3. However, the reliable onset of human vascular perfusion took 7 days based on our extensive observation in the past decade [9,15–18]. The timing inconsistency between human cell-mediated revascularization and beneficial cardiac remodeling indicated other therapeutic pathways might involve preserving cardiac functions in the early stage of myocardial infarction.

Gene expression profiling analysis in the early response of human vascular cells delivered in infarct hearts

To further elucidate the molecular aspects of the therapeutic benefits of ECFC+MSC delivery in GelMA-H, we conducted an exploratory transcriptional analysis using RNA sequencing (RNAseq). The infarct left ventricle tissues were harvested from GelMA-H and GelMA-S groups on day 2 post-MI to study the change of gene expression profiles in the early stage of treatments. In this study, the uninjured hearts served as controls.

Globally, there were 2,088 differentially expressed genes (DEGs) among three analyzed groups (**Figure 3A**). Hierarchical clustering analysis (**Figure 3B**) revealed the proximity between two post-MI groups (GelMA-H and GelMA-S) in comparison to uninjured controls. This pattern was confirmed by the principal component analysis (PCA; **Figure 3C**). We performed a Gene Ontology (GO) analysis of the DEGs between post-MI and control groups. The results showed that the top enriched GO categories due to infarct injury were associated with positive regulations of immunity, including inflammatory response, immune response, and chemotaxis. These findings suggest that the immune system was deeply involved in the early stage of the MI process.

To gain more insight into the transcriptional difference between GelMA-H and GelMA-S groups, we carried out the pairwise analysis of DEGs (**Figure 3E**). RNA profiling showed that, for analyzed genes, the expression of 137 was significantly upregulated (*P<0.001*; **Figure 3A**, GelMA-H vs. GelMA-S), whereas 617 was decreased (*P<0.001*). In the GelMA-H group, differentially expressed genes were clearly identified and grouped into GO terms (**Figure 3D**). Notably, we observed significant upregulation of several genes related to the neutrophil development and maturation (s100a8, s100a9, elane, ngp, mpo, camp, lef1, prg3, mmp3, and ms4a2). This was associated with significant upregulation of pathways implicated in immature neutrophil trafficking (sdf, cxcr4), hematopoiesis (gata1, hemgn), and immunomodulation (xxx) in GelMA-H vs. GelMA-S (**Figure 3E**, Volcano plot). Altogether, these data indicated the potential effects of human ECFCs+MSCs delivered in GelMA-H by shifting the innate immunity landscape, especially the recruitment and polarization of neutrophil subpopulations in the early stage of MI.

Recruitment of neutrophils with reparative phenotype in GelMA-H group

Our RNAseq data implicated the difference in neutrophil-related genes between GelMA-H and GelMA-S groups. In our previous study, we demonstrated that in vivo implantation of human vascular cells engaged a newly discovered, alternatively polarized neutrophil subpopulation, mediating vascular assembly and anastomosis [17]. Recently, these pro-regenerative neutrophils (herein termed **NR**; "R" for regeneration) were shown to actively orchestrate tissue repair in a variety of organs [19–29]. However, their role in post-MI healing is still unclear. Therefore, we focused on the polarization of the neutrophil subpopulation in the following analysis.

To examine the early-stage host cell infiltration post-MI, we analyzed the recruited mouse CD45+ leucocytes on day 2 in harvested infarct tissues and classified them into $Ly6G^{+}F4/80^{-}$ neutrophils or $Ly6G^{-}F4/80^{+}$ macrophages. Infiltration of lymphocytes was ignorable. As shown in **Figure 4B**, the presence of murine resident myeloid cells in the uninured myocardium was low. LAD ligation followed by either GelMA-H or GelMA-S treatment significantly recruited host myeloid cells, which is consistent with the induced innate immune response shown in our RNAseq analysis. Moreover, the GelMA-H group recruited more neutrophils and macrophages than the GelMA-S group, which is consistent with our previous observation that human ECFCs and MSCs engaged host myeloid cells through IL6, IL8, and CXCL1 secretion [17]. We further evaluated neutrophil subpopulations using flow cytometry and classified $Ly6G^{+}F4/80^{-}$ cells into naïve neutrophils (N0; CXCR2-), pro-inflammatory neutrophils (NI; $CXCR2^{+}CD206^{-}$), and pro-regenerative neutrophils (NR; $CXCR2^{+}CD206^{+}$; **Figure 4A and C**). Notably, the NI was the dominant subpopulation (83.27% of total neutrophils; **Figure 4D**) in the MI mice treated with GelMA-S. This data is

consistent with the Lindsey laboratory, which reported that pro-inflammatory neutrophils significantly mediated adverse cardiac remodeling in the first 7 days post-MI [22]. In contrast, NRs significantly increased to 57.66% of total neutrophils in the GelMA-H group, while NIs dramatically reduced to 18.02%, suggesting a shift toward NR polarization. The GelMA-H group also recruited more naïve neutrophils from the bone marrow than that in the GelMA-S group (24.32% vs. 13.31%). Together, these data revealed that improving acute human therapeutic cell retention favored a pro-regenerative host neutrophil polarization during the early stage of MI healing. Collectively in the literature, NRs were shown to promote healing by facilitating the resolution of inflammation and stimulating angiogenesis, cell proliferation, and ECM remodeling, which might explain parts of the beneficial effects observed in our GelMA-H group.

Human vascular cell interaction upregulated NR polarization factor TGFβ1

To further investigate how GelMA-H treatment modulated neutrophil subpopulations, we hypothesized that a prolonged interaction between human ECFCs and MSCs resulted in a release of paracrine factors that promote NR polarization. In contrast, these factors were absent in the GelMA-S group due to low cell retention. To validate this hypothesis, we collected conditioned media from ECFC+MSC coculture in GelMA hydrogel over 7 days in vitro (CM^{CO}). Murine naïve neutrophils isolated from bone marrow (BM) were cultured in the presence of CM^{CO} for 24 h. Increased NR polarization was evidenced by the upregulation of VEGFA and ARG1 gene expressions (**Figure 5A**). NR polarization was not detected in the culture of BM neutrophils in basal medium or conditioned media obtained from ECFC or MSC monoculture (CM^{ECFC} or CM^{MSC}).

To gain further insight into the NR polarization factors, we performed growth factor analyses of the conditioned media from the ECFC+MSC coculture (**Figure 5B-C**). Mixed conditioned media (1:1 mixture of CM^{ECFC}+CM^{MSC}; termed CM^{MIX}) obtained from separated ECFC and MSC monoculture served as a control. The secretion of several growth factors (most notably IGFBP-1, Endoglin, and TGFβ1) was induced in the ECFC+MSC coculture. Among them, TGF-β1 has been shown to promote NR polarization [17]. We confirmed at the RNA level that the ECFC+MSC coculture upregulated TGF-β1 expression only in ECFCs but not in MSCs (**Figure 5D-E**). Moreover, endothelial Notch signaling is known to mediate vascular cell interaction [17]. Deactivation of Notch by exposing ECFC+MSC coculture to the Notch inhibitor DAPT (a γ-secretase inhibitor) blocked the induction of TGF-β1 expression.

Next, to further elucidate the importance of TGF-β1 in NR polarization of BM neutrophils, we examined the effect of SB432542, a potent and selective inhibitor of the TGFβ type I receptor. We found that treatment with SB432542 impaired the NR polarization stimulated with CM^{CO}. Moreover, a supplement of recombinant human TGF-β1 in basal medium induced NR polarization. Collectively, these results suggested that NR polarization could be directly related to the state of the human vascular cells engrafted in infarct tissues. Notch-mediated interaction of ECFCs and MSCs could modulate the early innate immune landscape by increasing TGF-β1 secreted from endothelial compartments.

Neutrophil depletion cancelled the benefit of human vascular cell delivery and worsens cardiac function

To confirm the pivotal role of host neutrophils during the human vascular cell-mediated cardiac remodeling, we depleted circulating neutrophils in MI mice treated with GelMA-H. An anti-Gr1 or IgG control antibody was given by intraperitoneal injection every 2 days from two days before LAD ligation to post-operative day 6 (**Figure 6A**). We validated that 200 μg of anti-Gr1 antibody per injection per mouse every 2 days was shown to deplete circulating neutrophils whereas blood monocytes and lymphocytes were less affected (compared to IgG-treated control mice; **Figure 6B-C**). Treatment with anti-Gr1 antibody, compared to control IgG antibody, abolished the improved post-MI cardiac functions seen at days 3 and 7 after delivering human cells in GelMA-H (LVEF for **Figure 6D** and LVFS for **Figure 6E**); significantly reduced

myocardium thickness measured at day 7 (LVAW, **Figure 6F**). All three measured cardiac functions (LVEF, LVFS, and LVAW) in neutrophil-depleted mice decreased to the levels which is close to untreated or GelMA-S groups in Figure 2. Histological analysis of the myocardium revealed an increased percentage of fibrosis 7 days post-MI in neutrophil-depleted mice (**Figure 6**). The number of CD31-stained microvessels in healing infarcts of control and neutrophil-depleted mice was comparable (**Figure 6**). Consistent with a lower cardiac function, the myocardial thickness was thinner in infarcts of depleted mice (**Figure 6**). These findings suggest that depletion of neutrophils leads to excessive loss of myocardium that might explain the progressive worsening of cardiac function. Collectively, these results confirmed that host neutrophils are indispensable for the therapeutic effects exhibited by human vascular cells delivered in GelMA-H.

Discussion

In the present study we demonstrated that cell engraftment was significantly augmented by delivering human vascular progenitor cells in photocrosslinked GelMA hydrogel. An effective early intervention aimed at improving cell retention in the infarcted myocardium have important implications for the sustained presence of therapeutic cells to exhibit their therapeutic effects in the heart. Improved acute cell retention showed an impact on post-MI cardiac healing. Echocardiography and histology demonstrated that the delivery of ECFC+MPC in GelMA hydrogel provided a significant therapeutic benefit – slowing down the loss of cardiac functions, preserving viable myocardium, and preventing cardiac fibrosis. Human ECFCs and MSCs can form vascular networks after injection into a pathologic ischemic environment. However, ECFC+MPC-mediated vascular regeneration is not the sole mechanism leading to cardiac healing. Of interest, we observed a novel therapeutic benefit of human vascular cells which is modulating pro-regenerative neutrophil polarization.

Leukocyte infiltration in ischemic areas is a hallmark of MI. The inflammatory response appears as a double-edged sword because it is a protective attempt by the organism to initiate the healing process; however, inflammation itself can exacerbate scarring and loss of organ function. Overwhelming infiltration of innate immune cells has been shown to promote adverse remodeling and cardiac rupture. The successful resolution of inflammation appears as a key event in the repair of tissue damage, but little is known about the main mediators of this process in the infarcted cardiac tissue [30]. Neutrophils massively infiltrate the heart after MI, where they can promote both tissue repair and damage processes. Emerging evidence indicate that neutrophils can comprise two distinct subpopulations with different polarized phenotypes: a canonical pro-inflammatory phenotype (referred to as NI) and an alternative anti-inflammatory, pro-regenerative, phenotype (NR), resembling the well-established 'M1–M2' macrophage paradigm. Previous reports have suggested that cardiac neutrophils follow a temporal polarization pattern after myocardial infarction, switching from a proinflammatory NI to an anti-inflammatory NR profile [20,22,31–33]. However, this switch could take 5-7 days post-MI in rodent models, while excessive inflammatory cells had produced irreversible damages to surrounding myocardium [22]. Here we shown that an optimized delivery of human vascular progenitor cells rapidly engages the alternatively polarized NR host neutrophils in the ischemic tissue in as early as 2 days post-MI. NRs have been shown to actively contribute to the repair of various tissues, mainly through suppressing pro-inflammatory reactions to facilitate resolution, promoting M2 macrophage polarization, and stimulating angiogenesis, cell proliferation, and ECM remodeling [23,24,29,30]. Indeed, we reason that the early transition to NR-mediated inflammatory response is parts of the therapeutic mechanism achieved by human vascular cells delivered in infarcted hearts.

Over the years, numerous preclinical studies and several clinical trials have evidenced the feasibility of cell injection in treating heart diseases. However, a common hurdle is shared by all current delivery routes: low cell retention due to the "washout" effects of beating hearts. A myriad

of studies confirm that cell retention plays a crucial role in the success of cell-mediated cardiac repair. It is important for any delivery route to maintain donor cells in the recipient heart for enough time to send paracrine signals to surrounding damaged heart cells and repair them. Challenges associated with the in vivo cell delivery of currently available vehicles have limited clinical translation of this technology. Fibrin- or collagen-based hydrogels had been tested for intramyocardial cell delivery. However, the slow gelation process of these natural hydrogels failed to achieve a desirable cell retention. Gelatin methacryloyl (GelMA), which was synthesized in our previous studies as a photocrosslinkable, bioadhesive hydrogel, could address many of the limitations of available hydrogels for heart application. The goal of this proof-of-concept study was to evaluate the cardioprotective potential of human vascular-forming cells delivered in GelMA in a mouse model of MI[34].

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Figure 1

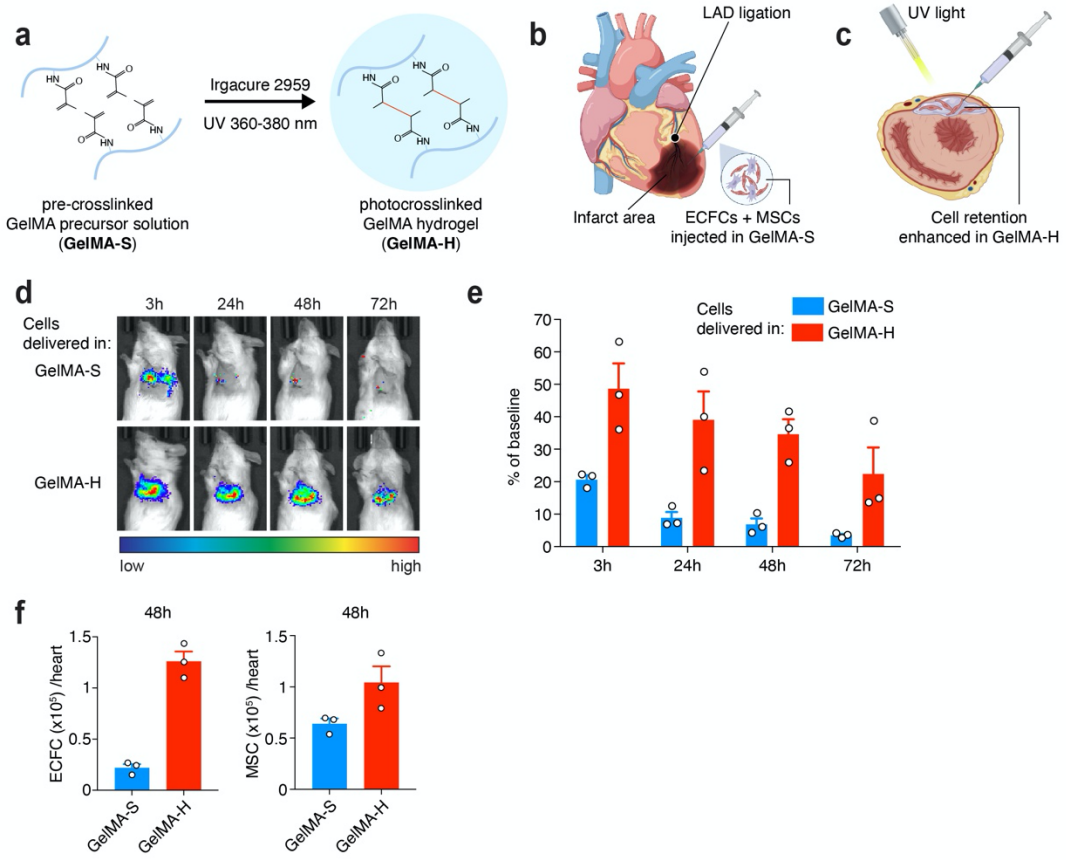

Figure 1. Enhanced intramyocardial cell retention by delivering human vascular cells in photocrosslinked GelMA hydrogel. (A) Schematic of photocrosslinking of GelMA from a precursor solution (GelMA-S) to a hydrogel matrix (GelMA-H) under UV irradiation. Free radicals generated by the photoinitiator initiated chain polymerization with methacryloyl substitution. (B) Schematic depicting intramyocardial cell injection. MI was surgically induced by LAD ligation. Human vascular cells (ECFCs + MSCs) resuspended in GelMA solution were injected in three locations in the infarct area. (C) UV light was applied on top of the infarct area to polymerize a cell-laden hydrogel (referred to as GelMA-H group). For control group, UV irradiation was omitted to maintain cells injected in precrosslinked GelMA solution (GelMA-S group). (D) Viable cell retention was measured by bioluminescence imaging of human ECFCs expressing a luciferase reporter. (E) Total bioluminescent signals in each animal's chest region were measured and compared to the baseline (measured by cell delivered to arrested mouse hearts). (F) Retention of human ECFCs or MSCs were quantified by flow cytometric analysis post 2 days. Data were displayed by counted cell number from each harvested MI heart. Statistical methods: ANOVA followed by Bonferroni's post-test analysis (E) and unpaired two-tailed Student's t-tests (F).

Figure 2

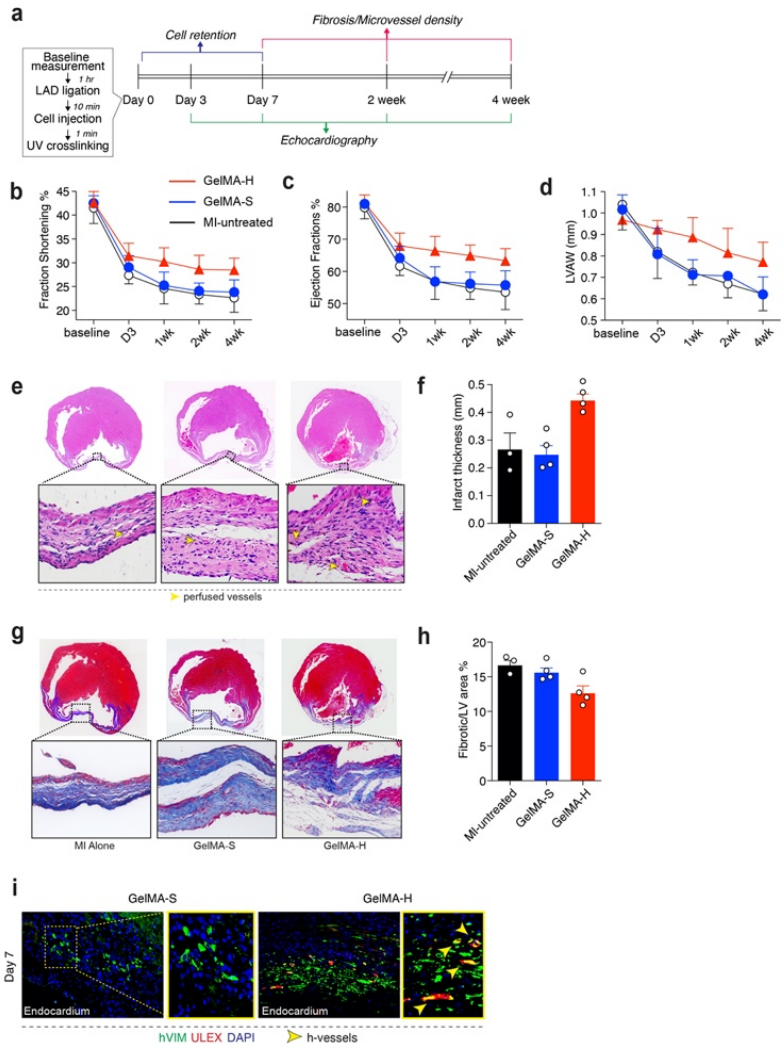

Figure 2. Human vascular cells delivered in GelMA hydrogel promoted beneficial cardiac remodeling. (A) Diagram of experimental schedule. (B-D) Echocardiography analysis for cardiac function. Echocardiography was performed on anesthetized mice at 3 days, and 1, 2, 4 weeks post-MI following cells delivered in GelMA-H or GelMA-S (n=6 for each group). Mice received LAD ligation, but no cell injection (MI-untreated) served as controls (n=6). Baseline data were obtained from mice within 1h before performing LAD ligation. (B) Left ventricular fractional shortening (LVFS). (C) Left ventricular ejection fraction (LVEF). (D) Left ventricular anterior wall thickness (LVAW). (E) Representative heart horizontal panoramic views (upper) and microscopic images (lower) of myocardial sections stained with H&E. Scale bars, xxx mm. (F) The LV wall thickness in infarct areas quantified by H&E sections. (G) Representative heart sections stained with Masson's trichrome. Scale bars, xxx mm. (H) The percentage of fibrotic areas in the total LV crossections quantified by Masson's trichrome staining. (I) Histological analysis of infarct myocardium on day 7 observed the formation of perfused human vasculature only when cells were delivered in GelMA-H group, but not in GelMA-S. Human microvessels identified by immunostaining for human-specific vimentin (hVIM) and UEA-1 lectin binding. Scale bars, xxx mm. (J) Total perfused human microvessel density quantified in infarct myocardium. Statistical methods: ANOVA followed by Bonferroni's post-test analysis (B-D) and unpaired two-tailed Student's t-tests (F, H, J). Data present mean $\pm$ SD. Numbers above bars are P values.

Figure 3

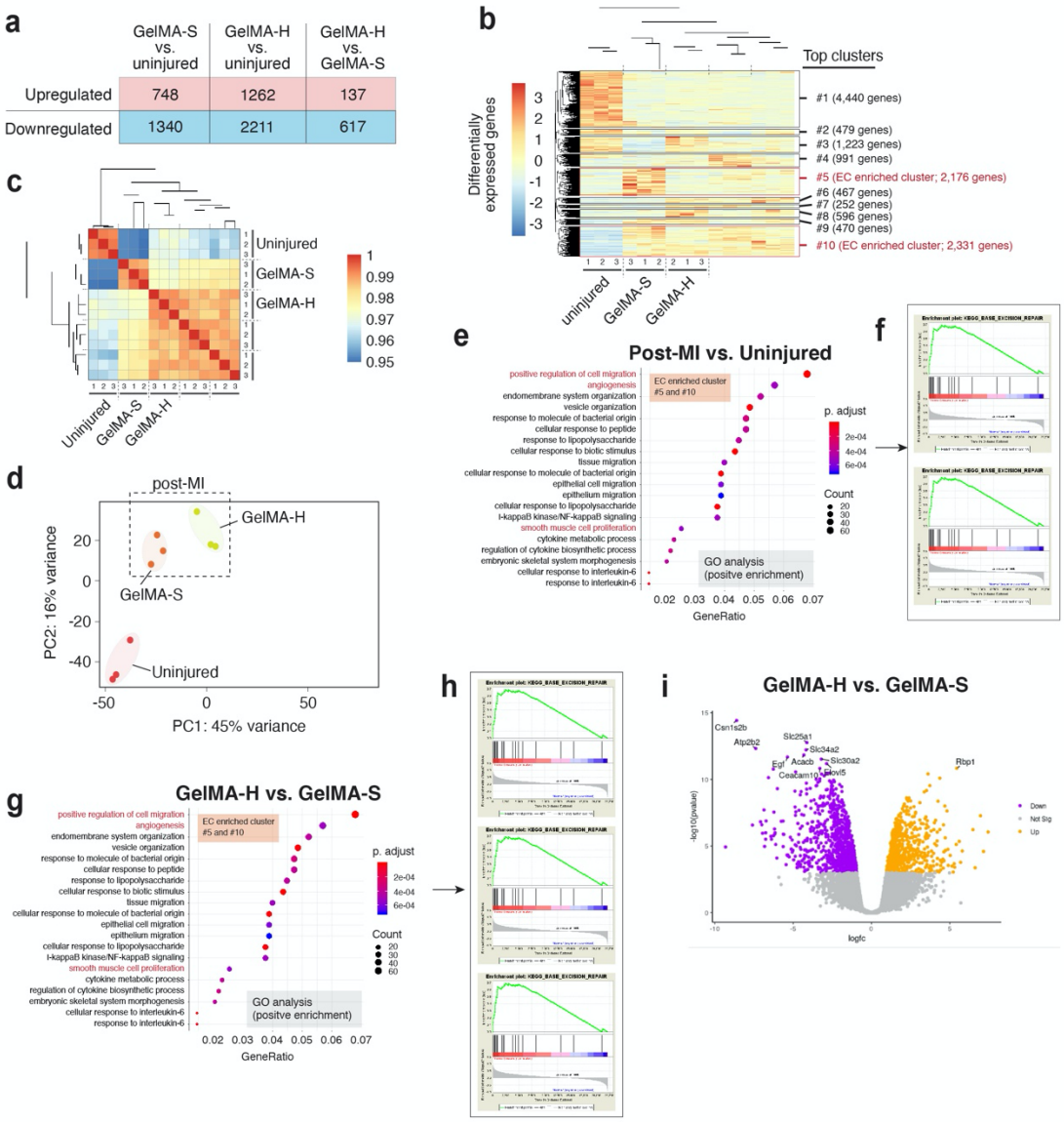

Figure 3. Gene expression profiling analysis in the early response of human vascular cells delivered in infarct hearts.

Figure 4

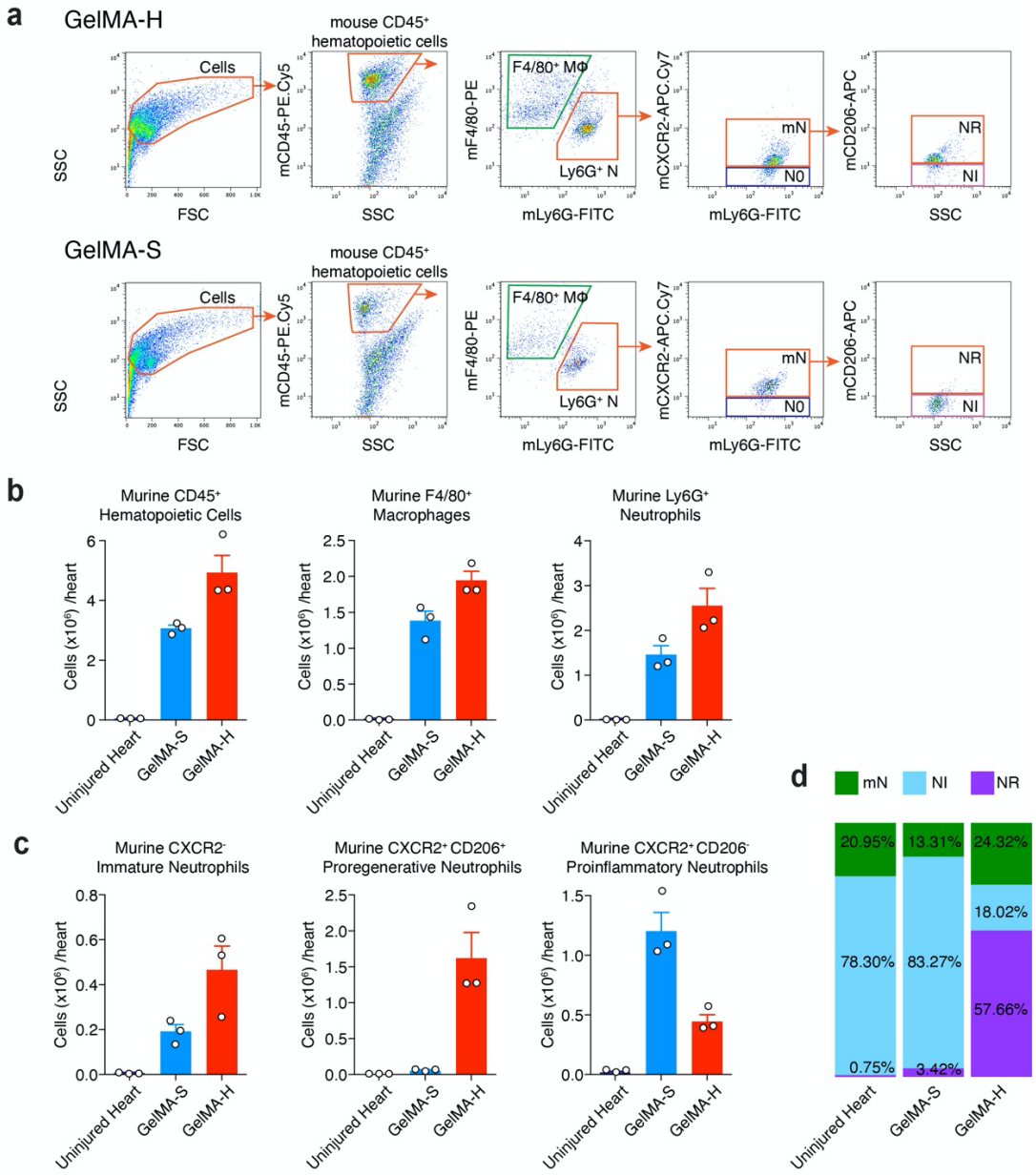

Figure 4. Recruitment of neutrophils with reparative phenotype in GelMA-H group. (A)

Figure 5

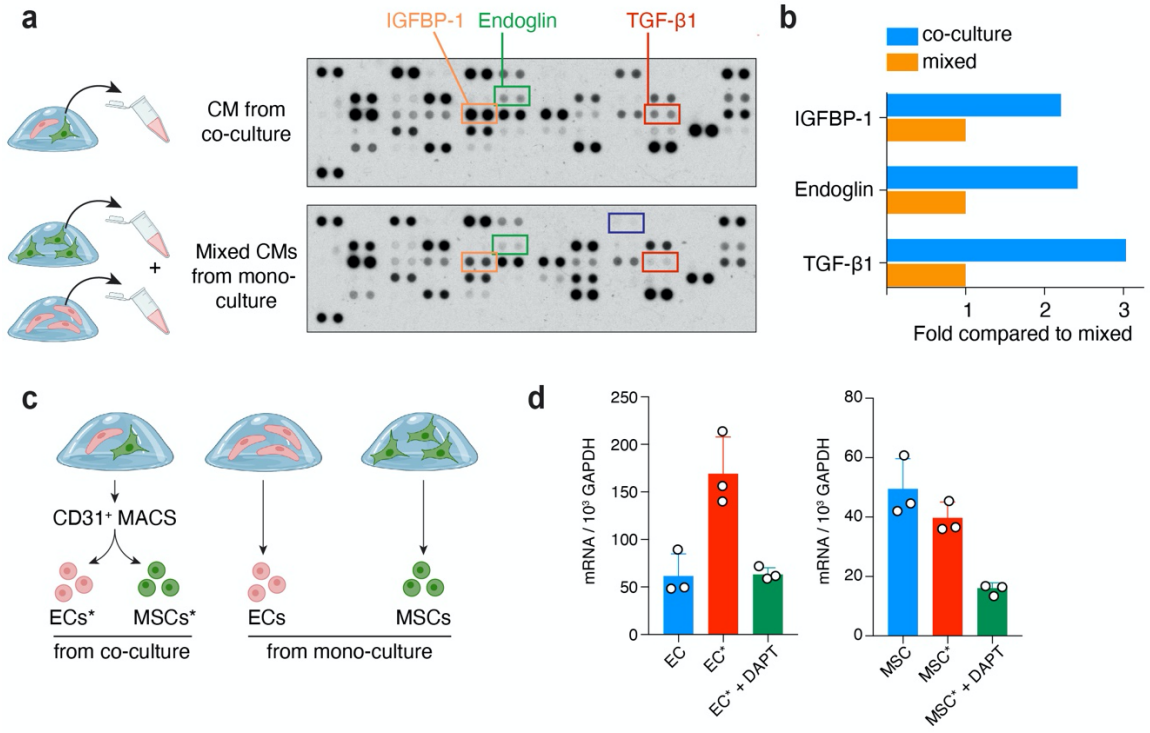

Figure 5. Human vascular cell interaction upregulated NR polarization factor TGFb1. (A)

Figure 6

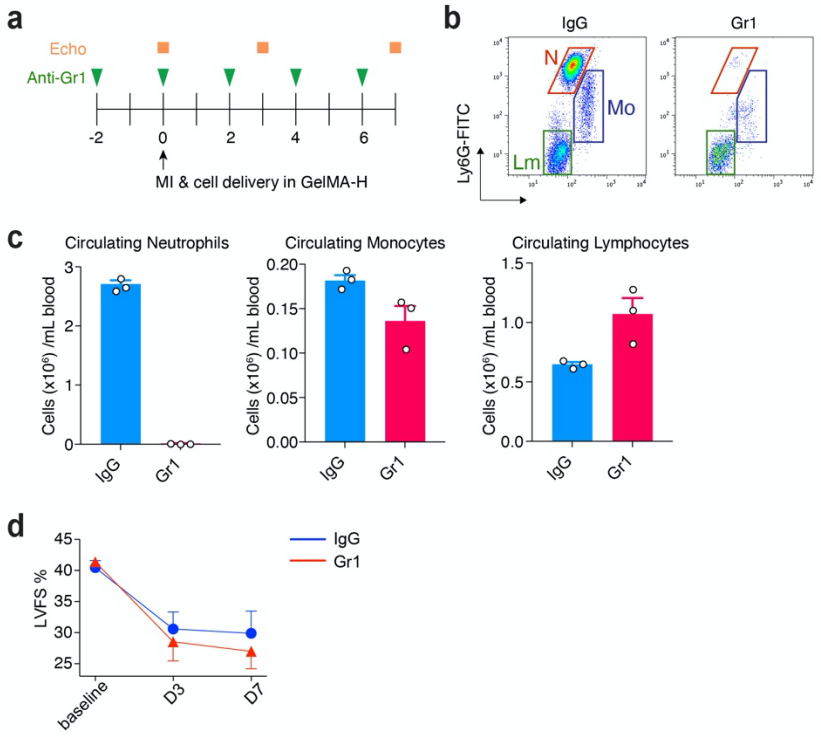

Figure 6. Neutrophil depletion cancelled the benefit of human vascular cell delivery. (A)