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

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Abstract

The success of vascular progenitor cell transplantation to treat myocardial infarction (MI) is primarily limited by the low retention/engraftment of the delivered cells. Contracting myocardium actively pushes therapeutic cells injected in liquid vehicles out of the heart, resulting in low cell retention and poor therapeutic outcome. A clinically applicable biomaterial to improve cell retention is arguably needed to enable optimization of intramyocardial cell delivery. In this study, we used the MI model by ligating the left anterior descending arteries in mice. We developed a novel therapeutic cell delivery method for MI treatment based on the photocrosslinkable GelMA hydrogel. Human endothelial colony-forming cells (ECFCs) and mesenchymal stem cells (MSCs), a combination of vascular progenitor cells known to form functional vascular networks after transplantation in vivo, were injected in GelMA precursor solution into the infarcted zone of mouse hearts. The injected area was immediately illuminated by UV light to produce an in-situ photocrosslinked, cell-laden hydrogel. We demonstrated this approach significantly enhanced the therapeutic cell retention in beating hearts and achieved a sustained presence of therapeutic cells to exhibit their therapeutic effects. The improved acute cell retention translated to a long-term beneficial post-MI cardiac healing, including stabilizing cardiac functions, preserving viable myocardium, and preventing cardiac fibrosis. Furthermore, based on this technical breakthrough, we reported for the first time that the mechanism of vascular cell delivery promotes post-MI cardiac healing by polarizing pro-regenerative neutrophils. TGFβ produced by endothelial cells engrafted with proper perivascular interaction polarized the recruited bone marrow neutrophils toward a pro-regenerative phenotype, establishing a pro-regenerative microenvironment that favorites post-MI cardiac healing. Depletion of host neutrophils canceled the therapeutic benefits of cell delivery in ischemic hearts, indicating the non-inflammatory, pro-regenerative neutrophils are indispensable mediators of beneficial cardiac remodeling. Taken together, we demonstrated a GelMA hydrogel-based human vascular progenitor cell therapy for the clinical translational potential to treat acute MI.

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]. MI patients suffer from decreased cardiac output, resulting in a downward spiral leading to congestive heart failure. In clinical, the 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, 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]. For years, considerable effort has been focused on delivering therapeutic genes, growth factors, and cells to promote post-myocardial infarction healing. However, these strategies are insufficient to a certain extent because of the complexity of the ischemic, hyper-inflammatory microenvironment in the infarcted myocardium.

While the human heart is considered a terminally differentiated organ, adult cardiac regeneration by endogenous repair mechanisms is unfeasible with current medical options. Over the last decade, many investigators have tested cell-based therapies to prevent adverse ventricular remodeling and promote 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. First, cardiac patches or cell sheets consisting of pluripotent stem cell-derived cardiomyocytes were transplanted to reinforce or replace the injured myocardium aiming to rebuild the loss of heart walls. These cardiac patches are mainly manufactured by seeding cells in biomaterial scaffolds and preconditioned in a bioreactor before surgical implantation. The second approach injected therapeutic cells directly into the infarcted areas to mitigate the detrimental impact of MI. In human patients, delivery of cells could be achieved by intramyocardial or intracoronary injection routes through a catheter-based minimally invasive surgical approach, which allows the early therapeutic intervention in the acute MI phase [9]. Examples of adult stem/progenitor cells that have been tested include cardiosphere-derived cells, bone marrow-derived mononuclear cells, endothelial progenitor cells, and mesenchymal stem cells. Except for cardiomyocytes, the long-term engraftment of injected cells was shown to be unnecessary to exhibit their therapeutic effects. Accumulating evidence suggested non-myocyte cell types can provide beneficial effects 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 therapeutic strategy for ischemic cardiomyopathy. Some studies have focused on delivering 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. 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 a modest reduction in adverse ventricular remodeling. The quantitative analysis showed a rapid loss of human cells shortly after myocardial injection, suggesting the lack of sufficient cellular retention could be the major reason for the 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.

Low cell retention and engraftment are the major obstacles to achieving a significant functional benefit in MI, irrespective of the cell type used. Beating hearts actively push therapeutic cells injected in liquid vehicles out of the myocardium, resulting in low acute cell retention between 0.5-10% across several studies with different cell types, animal models, and delivery routes. To address this problem, several biomaterials for enhancing cell engraftment have been tested in the last decade. 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. Cell-laden GelMA formulation can polymerize rapidly (10 seconds upon exposure to UV light in the presence of a photoinitiator) in myocardial tissues without compromising the therapeutic cell viability. 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. Importantly, enhanced cell retention allows us to study and discover the early beneficial effects of human vascular cells in MI for the first time. We found that the enhanced retention of human ECFCs and MSCs enabled the perivascular cell-cell interactions to upregulate endothelial TGFβ expression. TGFβ 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 (**Figure 1**). MI was induced by permanent ligation of the left anterior descending (LAD) arteries (**Figure S1A** and **Video S1**, Supporting Information) in 10-12-week-old SCID mice. 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 infarcted myocardium within 10 min of LAD ligation. The total injection volume per heart is 100 μL. We first demonstrated that this injection volume is sufficient to cover the infarcted zone (**Figure S1B**). We then determined 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**). The general safety of short UV illumination by our UV lamp had been evaluated in our previous study [10]. Our working range of UV light exposure (10 - 45 seconds) was deemed sufficiently safe for both injected cells and surrounding tissues [10]. 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 hydrogel following injection into infarcted zone (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°C and in contact with blood or tissues) if not crosslinked by UV [11]. Since the cardiac contraction had been proved to be the primary culprits in the early washout of cells from the injection site[12], 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 20.6 ± 2.3% (**Figure 1D-E**), a low retention rate in accordance with previous studies that quantified the efficiency of cell delivery in plain PBS [12–14]. 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 improved to 48.6 ± 13.6% (3

h, $p < 0.05$ 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 28.8% from 48.6 ± 13.6% at 3h to 34.6 ± 8% at 72h). In comparison, the injected cells in GelMA-S lost rapidly within the initial 24 hours (reduced 67% from 20.6 ± 2.3% at 3h to 6.8 ± 3.1% at 48h).

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**; p value = 0.0005 for ECFCs and 0.0728 for MSCs). Compared to the initial injected cell numbers, the GelMA-H group preserved 25.2% of viable ECFCs and 20.8% of MSCs, while the GelMA-S group only kept 4.4% of ECFCs and 12.8% of MSCs. 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 [15]. 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 acute phase (72h) of therapeutic cell engraftment.

Human vascular cells delivered in GelMA hydrogel promoted beneficial cardiac remodeling

To determine whether the improvement in acute cell retention would impact post-MI cardiac healing, we used echocardiography to evaluate the cardiac functions (**Figure 2 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;** 30.3 ± 2.9%, 24.6 ± 3.2%, 25.2 ± 2.8% for LVFS; 66.4 ± 4.5, 56.9 ± 5.6, 56.8 ± 4.6 for LVEF, respectively). 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 (increased 20.2%, 18.7%, and 19.7% for LVFS; 16.9%, 17.4%, and 17.1% for LVEF). Both untreated or GelMA-S-treated groups showed a continuous decline in LVEF and LVFS from day 3 to 28 post-MI (MI-untreated: reduced 17.6% for LVFS and 13.4% for LVEF; GelMA-S: reduced 19.6% for LVFS and 14.3% for LVEF), 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 (GelMA-H: reduced 9.5% for LVFS and 6.7% for LVEF).

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 after 4 weeks (**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;** 0.44 ± 0.05 mm vs. 0.25 ± 0.06 mm, $^*p < 0.01$). Immunofluorescent staining confirmed the higher survival of cardiomyocytes (cTnT+) within the infarct area of GelMA-H mice. Fluorescent image assessment demonstrated that the number of human microvessels in GelMA-H-treated hearts was significantly higher in the infarcted area than in the GelMA-S group (**Figure 2G-H**). 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 [16]. 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 2G**). 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 2H**). 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 [10,17–20]. 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 S4A**). Hierarchical clustering analysis (**Figure S4B**) 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 S4C).** To gain more insight into the transcriptional difference between GelMA-H and GelMA-S groups, we carried out the pairwise analysis of DEGs. RNA profiling showed that, for analyzed genes, the expression of 137 was significantly upregulated (**Figure S4A**, GelMA-H vs. GelMA-S), whereas 617 was decreased. The volcano plot (**Figure S4D**) shows the up- and down-regulated genes comparing GelMA-H group to GelMA-S group. 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 genes implicated in immature neutrophil trafficking (sdf, cxcr4), hematopoiesis (gata1, hemgn), and immunomodulation (il4, il10, tgfb) in GelMA-H vs. GelMA-S (**Figure S4D-E**). 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. However, we noticed that the data variation between samples within GelMA-H group is high. This problem is probably due to the difficulty to perform in vivo mouse MI studies identically between animals. The minor differences in the MI surgical procedure, cell injection, and tissue harvesting may result in the data variation amplified by RNAseq.

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 [19]. Recently, these pro-regenerative neutrophils (herein termed NR; "R" for regeneration) were shown to actively orchestrate tissue repair in a variety of organs [21–31]. 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 3B**, 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 [19]. 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 3A and C**). Notably, the NI was the dominant subpopulation (83.27% of total neutrophils; **Figure 3D**) 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 [24]. 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 4A**). 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 4B-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 [19]. We confirmed at the RNA level that the ECFC+MSC coculture upregulated TGF-β1 expression only in ECFCs but not in MSCs (**Figure 4D-E**). Moreover, endothelial Notch signaling is known to mediate vascular cell interaction [19]. Deactivation of Notch by exposing ECFC+MSC coculture to the Notch inhibitor DAPT (a γ-secretase inhibitor) blocked the induction of TGF-β1 expression. 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 5A**). 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 5B-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 5D** and LVFS for **Figure 5E**); significantly reduced myocardium thickness measured at day 7 (LVAW, **Figure 5F**). 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 5**). The number of CD31-stained microvessels in healing infarcts of control and neutrophil-depleted mice was comparable (**Figure 5**). Consistent with a lower cardiac function, the myocardial thickness was thinner in infarcts of depleted mice (**Figure 5**). 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

An effective early intervention to achieve sufficient cell retention in the infarcted myocardium has important implications for the sustained presence of therapeutic cells to exhibit their therapeutic effects. In the present study, we demonstrated that beneficial cell retention in MI hearts was significantly augmented by delivering human vascular progenitor cells in the photocrosslinked GelMA hydrogel. In contrast, cells injected in GelMA precursor solution without UV illumination lose rapidly from the beating hearts. Notably, the improved acute cell retention showed an impact on post-MI cardiac healing. Echocardiography and histological examination demonstrated that the delivery of ECFC+MSC in GelMA hydrogel provided a significant therapeutic benefit – slowing down the loss of cardiac functions, preserving viable myocardium, and preventing cardiac fibrosis. As expected, human ECFCs and MSCs formed perfused vascular networks after being engrafted 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 through modulating pro-regenerative neutrophil polarization.

Leukocyte infiltration in ischemic areas is a hallmark of MI. The inflammatory response appears a double-edged sword because it is a protective attempt by the organism to initiate the healing process; however, prolonged inflammation 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 critical event in the repair of tissue damage, but little is known about the primary mediators of resolution in the infarcted cardiac tissue [32]. Neutrophils massively infiltrate the heart after MI, where they can promote both tissue repair and damage processes. Emerging evidence indicates 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 MI, switching from a pro-inflammatory NI to an anti-inflammatory NR profile [22,24,33–35]. However, this switch could take 5-7 days post-MI in rodent models, while excessive inflammatory cells had produced irreversible damage to the surrounding myocardium [24]. Here we showed that an optimized delivery of human vascular progenitor cells rapidly engages the alternatively polarized host neutrophils in the ischemic tissues 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 [19,22,23,30]. Indeed, we reason that the early transition to NR-mediated regenerative response is part of the therapeutic mechanism achieved by human vascular cells delivered in infarcted hearts.

Transforming growth factor β (TGFβ) has been shown to promote the NR phenotype. NRs in the injury site derive from the TGFβ-mediated polarization of naïve neutrophils (termed N0) recruited from the bone marrow (BM). Blocking TGFβ signaling results in a depletion of the NR subpopulation and leads to a loss of vascularization [19]. We have shown that human ECFC+MSC implanted in the subcutaneous space of immunodeficient mice can form robust vascular networks anastomosed with host circulation within a week [10,20,36,37]. The vascularization of human vascular cells required host neutrophils expressing TGFβ receptor 2 (TGFBR2) [19]. Human ECFC+MSC implanted in neutrophil-depleted animals (by sub-lethal radiation) completely failed to vascularize; however, an adoptive transfer of bone marrow (BM)-derived neutrophils from unirradiated wild-type donor mice fully rescued vascularization [19]. Of note, the adoptive transfer of BM-derived neutrophils lacking TGFBR2 (from *Tgfbr2*$^{-/-}$ mice; *LysM-Cre*::*Tgfbr2/loxP*) failed to rescue vascularization, which underscored the critical role of TGFβ-TGFBR2 signaling in the generation of functional NRs [19]. Here, we further confirmed that the successfully engrafted human ECFCs are a critical source of active NR-polarizing factors. We found that the conditional medium from ECFC+MSC coculture in a GelMA hydrogel strongly promoted NR polarization, which is evidenced by the up-regulation of NR signature genes, *Arg1*, *Vegfa*, and *Il4*, in mouse BM neutrophils. ECFC-derived TGFβ1, which is up-regulated only in the presence of MSCs, could be a key mediator in our study. Furthermore, while the endothelial-perivascular cell interaction was disrupted by blocking Notch-mediated signaling, ECFCs failed to up-regulate TGFβ1 expression. Taken together, we postulate that the beneficial effect of delivering human vascular cells in GelMA hydrogel is partally due to promoting ECFC-derived TGFβ1 secretion and sequentially accelerate the engagement of host NRs, resulting in a pro-regenerative microenvironment that favorites post-MI cardiac healing.

Over the years, numerous preclinical studies and clinical trials have evidenced the feasibility of cell injection in treating heart diseases [38]. Systemic infusion of cells has been shown to be inefficient at delivering cells to the heart. It could even cause adverse effects when the injected cells accumulate in other organs, such as the lungs [39]. For local intracoronary injection, injected cells need to undergo transendothelial migration to reach the infarcted areas [40]. Studies have shown the risks of forming plugs when transplanted cells pass through narrow capillaries, which have diameters similar to cells [38,41]. Thus, those blocked cells become the barrier that prevents other cells from traveling from the coronary vessels to the ischemic zone.

As one of the most straightforward routes to deliver cells to the heart, the intramyocardial injection has been popularly applied in preclinical animal studies and clinical trials [42–45]. With a needle or catheter, an intramyocardial injection can precisely deliver a sustainable amount of therapeutic cells into pathological tissues in a short period of time. The intramyocardial injection can be performed through puncturing the myocardium from the epicardial side with a needle and injecting cells [38]. The epicardial intramyocardial injection is common in small animal models and was used in this study. However, this method requires open-chest surgery to expose the heart, which limits its clinical application. The endocardial intramyocardial injection also requires penetration of the myocardium, but instead from the inside of the ventricular wall. In human patients, this method can be achieved by a long catheter threaded through the peripheral blood vessels into the left ventricle, where the cells are injected through the endocardium. In general, this approach is less invasive because of no need for open-chest surgery. For this reason, the

endocardial intramyocardial injection possesses a higher translational potential. However, it is also very challenging to perform in small animal models.

A common hurdle is shared by all current delivery routes: low cell retention due to the “washout” effects of beating hearts. Given that the heart is a contractile pump, the beating of cardiomyocytes squeezes the narrow space in the myocardium and pushes transplanted cells in fluid medium out through the needle track or damaged blood vessels [38]. Many studies had reported a rapid loss of >80% of injected cells within hours after delivery [44,46]. Besides blood flow, the cells can be washed out of the heart by lymphatic drainage. In addition to physical washout from the heart, transplanted cells also lose over time through cell death before making a therapeutic benefit on the injured cardiac tissue. Inflammatory cells and damaged cardiomyocytes in the infarcted tissue can release cytokines and reactive oxygen species, which results in transplanted cell apoptosis [47]. Furthermore, the infarcted microenvironment is deficient in oxygen and nutrient supply. As a result, many transplanted cells die in these unfavorable living conditions.

A myriad of studies confirms that cell retention plays a crucial role in the success of the cell-mediated cardiac repair. Any delivery route needs to maintain donor cells in the recipient's 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. To improve the retention performance of therapeutic cells in the heart, generations of scientists have been focusing on bioengineering strategies. As the most thriving strategy to alter transplanted cells' low retention, biomaterials have been innovated in the past decade aiming at protecting cells from washout in the heart [48,49].

Several injectable hydrogels have been designed with different base materials and cell type combinations [50]. Previously, synthetic polymers and nanogels were used to encapsulate cells for delivery into mouse and swine models of MI to demonstrate their safety and efficacy [51–53]. Notwithstanding the benefit of these new materials, the translational usages of synthetic materials faced obstacles in clinical due to the limited data on the long-term biocompatibility of implanted materials and their metabolites. For this reason, various natural extracellular matrix (ECM) products have been extensively tested for intramyocardial cell injection, including fibrin- or collagen-based hydrogels [12]. However, the slow gelation process of these natural hydrogels failed to achieve desirable cell retention. Accordingly, improving the properties of naturally occurring ECMs has become an alternative approach. This can be achieved by the chemical functionalization of proteins, an approach that was proposed precisely to improve the usability of biomaterials [54–57]. Here, we used Gelatin methacryloyl (GelMA) synthesized in our previous studies by adding methacrylate groups to the amine-containing side-groups of gelatin, which becomes a photocrosslinkable, bioadhesive hydrogel [18,58]. As a result, GelMA hydrogels carry advantages that are representative characters of both natural and synthetic biomaterials. GelMA is highly soluble in phosphate-buffered saline and can be prepared as a precursor solution (5-20% w/v) at room temperature. Therapeutic cells can be easily suspended in the GelMA precursor solution for the needle- or catheter-based injection. Notably, the photocrosslinking reaction produces a GelMA hydrogel within 10 seconds of UV illumination without compromising encapsulated cell viability. Consequently, the quick gelation process of GelMA hydrogel effectively stops the washing out of cells injected in the hearts, resolving the main physical problem of low cell retention. Furthermore, we have shown that GelMA hydrogel possesses excellent biocompatibility. In our previous studies, GelMA hydrogel provided an ideal microenvironment for the engraftment and vascularization of human vascular progenitor cells both in vitro and in vivo in immunodeficient mice [10,18]. In particular, GelMA contains gelatin as its backbone, which provides cell-responsive characteristics such as the provision of appropriate cell adhesion sites and proteolytic degradability. Degraded products of GelMA hydrogel are simply gelatin peptides, which are parts of natural ECM components and are non-cytotoxic or non-immunogenic to the hearts [58].

The intensity needed to photocrosslink GelMA hydrogels transmyocardially was analyzed for safety. Transmyocardial exposure to our UV light spectrum (UVA-visible: 320-500 nm) had little observable effect on the human cells and surrounding mouse tissues. We have tested previously;

even exposing our human cells or rodent skins to prolonged UV illumination (120 seconds) showed minimal effects on cell apoptosis [10]. In this study, we achieved sufficient transmyocardial GelMA photocrosslinking with only 10 seconds of UV illumination. Therefore, our working range of UV light exposure was deemed sufficiently safe. Alternatively, GelMA can be formulated to form a hydrogel with visible light-responsive photoinitiators to eliminate the usage of UV light [59]. However, the crosslinking time using visible light is significantly longer than UV light [60]. Notably, our group demonstrated that UV light illumination through a fiber-optic system could be used together with the catheter-mediated application of the photocurable polymer patch to fix septal defects in the beating hearts of pigs [61]. Patches attached to the heart by UV crosslinking showed similar inflammatory response as sutures, with 100% small animal survival, indicating safety and applicability of UV system in minimally invasive delivery in hearts.

In situ photocrosslinked GelMA hydrogel functions as a cell delivery vehicle in our study. We had shown previously the implantation of GelMA hydrogel alone containing no cell provoked minimal host cell infiltration, suggesting GelMA is a relatively inert material [18]. A recent study by Ptaszek et al. applied GelMA hydrogel on top of the epicardial surface of the mouse heart at the time of experimental MI [62]. Mice treated with these bioadhesive GelMA patches displayed improved post-MI survival rates and left ventricular contractile function, raising the opposability that our GelMA hydrogel may benefit cardiac healing by providing mechanical support. However, we compared our intramyocardial GelMA-cell injection to their GelMA bioadhesive application and found a significant difference between the two experimental conditions. In our condition (by using minimal UV exposure time), the mechanical properties of GelMA hydrogel are relatively low compared to their protocol (elastin modulus, ~2 kPa vs. ~70 kPa). We reason that the disparity came from the fundamental difference in purpose between the two studies. We choose GelMA crosslinking condition based on our previous study to optimize the viability and vasculogenic potential of encapsulated human ECFC+MSC. We determined that a soft GelMA hydrogel (in the range between 2 to 5 kPa) is permissive for the formation of functional vascular networks. In contrast, Ptaszek's study aimed to apply bioadhesive GelMA to reenforce the loss of mechanical strength of the infarcted myocardial wall. They achieved a stiffer GelMA patch through a significant longer (up to 4 min) UV illumination. For this purpose, we anticipated that our cell-laden GelMA hydrogel injected intramyocardially is unlikely to provide compatible mechanical support shown in Ptaszek's report.

We acknowledge the limitations of this study. One major limitation came from using immunodeficient mice due to the need to transplant human cells. The SCID mice used in this study have a genetic immune deficiency that affects their B and T cells while their myeloid cell lineages remind normal. We didn't notice dysregulated neutrophil behaviors in the absence of functional B and T immunity compared to other studies of neutrophil recruitment post-MI in immunocompetent mice [22,24]. Moreover, to validate the dependency of graft vascularization on host neutrophils in immunocompetent hosts, we transplanted mouse autologous endothelial cells and MSCs isolated from C57BL/6 mice to syngeneic recipients [19]. In brief, we demonstrated that neutrophil participation was critical for both syngeneic (immunocompetent recipients) and xenograft (immunodeficient recipients) cell engraftment; and lymphocyte presence in the grafts was minimal. However, with the limited preclinical data, we cannot fully predict the outcome in human patients due to the difference in the immune system between humans and rodents.

The other limitation of the mouse MI model is the difficulty of performing more precise modeling and analysis of cardiac functions. First, we induced MI by permeant ligation of LAD in mouse hearts. More clinically relevant ischemic damage is the cardiac ischemia-reperfusion injury (IRI) [16]. However, performing IRI in mouse hearts is known to be challenging and results in significant variations and problems of reproducibility in animal test data. Second, the most likely intramyocardial cell delivery route in clinical is through a minimally invasive catheter procedure to penetrate the endocardial side. However, this procedure is extremely difficult to be performed in mice and rats. Third, the analyses of cardiac functions in mice are limited to imaging-based measurements (echocardiography and magnetic resonance imaging) for live animals or histological examination for end-point experiments. By incorporating large animals in the future

study, more precise analysis like the invasive hemodynamics with PV loops for the long-term following is ideal for providing a more comprehensive evaluation.

In summary, we have developed a novel therapeutic cell delivery method for MI treatment. Through the intramyocardial injection of cells resuspend in GelMA precursor solution followed by transmyocardial UV illumination, an in-situ photocrosslinked, cell-laden hydrogel is delivered effectively inside the ischemic myocardial tissues. Our approach maintained high viable cell retention, providing a significant advantage over cells injected in liquid or unmodified ECM gels. Furthermore, sustainable retention of human vascular progenitor cells allowed us, for the first time to investigate the therapeutic mechanism of vascular-forming cell formula post-MI. Importantly, we determined the engagement of pro-regenerative neutrophils through TGFβ signaling is indispensable for the beneficial cardiac remodeling and stabilization of cardiac functions post-MI. This proof-of-concept study warrants further investigations, including those related to i) scaling-up for large animal tests, ii) integrating with the catheter-mediated injectate and UV delivery, and iii) potential modification to include third cell types, for example, iPSC-derived cardiomyocytes. We envision that our research provides an opportunity to generate off-the-shelf cell-based therapy with the clinical translational potential to treat acute myocardial infarction.

Reference

1. Reed, G. W., Rossi, J. E. & Cannon, C. P. Acute myocardial infarction. *Lancet* 389, 197–210 (2017).

2. Members, W. G. *et al.* Heart Disease and Stroke Statistics—2012 Update. *Circulation* 125, e2–e220 (2012).

3. Westman, P. C. *et al.* Inflammation as a Driver of Adverse Left Ventricular Remodeling After Acute Myocardial Infarction. *J Am Coll Cardiol* 67, 2050–2060 (2016).

4. Stone, G. W. *et al.* Comparison of Angioplasty with Stenting, with or without Abciximab, in Acute Myocardial Infarction. *New Engl J Medicine* 346, 957–966 (2002).

5. Montalescot, G. *et al.* Platelet Glycoprotein IIb/IIIa Inhibition with Coronary Stenting for Acute Myocardial Infarction. *New Engl J Medicine* 344, 1895–1903 (2001).

6. Katarzyna, R. Adult Stem Cell Therapy for Cardiac Repair in Patients After Acute Myocardial Infarction Leading to Ischemic Heart Failure: An Overview of Evidence from the Recent Clinical Trials. *Curr Cardiol Rev* 13, (2017).

7. Pavo, N. *et al.* Cell therapy for human ischemic heart diseases: Critical review and summary of the clinical experiences. *J Mol Cell Cardiol* 75, 12–24 (2014).

8. Shah, V. K. & Shalia, K. K. Stem Cell Therapy in Acute Myocardial Infarction: A Pot of Gold or Pandora's Box. *Stem Cells Int* 2011, 536758 (2011).

9. Dib, N., Khawaja, H., Varner, S., McCarthy, M. & Campbell, A. Cell Therapy for Cardiovascular Disease: A Comparison of Methods of Delivery. *J Cardiovasc Transl* 4, 177–181 (2011).

10. Lin, R.-Z., Chen, Y.-C., Moreno-Luna, R., Khademhosseini, A. & Melero-Martin, J. M. Transdermal regulation of vascular network bioengineering using a photopolymerizable methacrylated gelatin hydrogel. *Biomaterials* 34, 6785–6796 (2013).

11. Yue, K. *et al.* Synthesis, properties, and biomedical applications of gelatin methacryloyl (GelMA) hydrogels. *Biomaterials* 73, 254–271 (2015).

12. Terrovitis, J. *et al.* Noninvasive Quantification and Optimization of Acute Cell Retention by In Vivo Positron Emission Tomography After Intramyocardial Cardiac-Derived Stem Cell Delivery. *J Am Coll Cardiol* 54, 1619–1626 (2009).

13. Fukushima, S. *et al.* Direct Intramyocardial But Not Intracoronary Injection of Bone Marrow Cells Induces Ventricular Arrhythmias in a Rat Chronic Ischemic Heart Failure Model. *Circulation* 115, 2254–2261 (2007).

14. Robey, T. E., Saiget, M. K., Reinecke, H. & Murry, C. E. Systems approaches to preventing transplanted cell death in cardiac repair. *J Mol Cell Cardiol* 45, 567–581 (2008).

15. Melero-Martin, J. M. *et al.* In vivo vasculogenic potential of human blood-derived endothelial progenitor cells. *Blood* 109, 4761–4768 (2007).

16. Kang, K.-T., Coggins, M., Xiao, C., Rosenzweig, A. & Bischoff, J. Human vasculogenic cells form functional blood vessels and mitigate adverse remodeling after ischemia reperfusion injury in rats. *Angiogenesis* 16, 773–784 (2013).

17. Kang, K.-T., Lin, R.-Z., Kuppermann, D., Melero-Martin, J. M. & Bischoff, J. Endothelial colony forming cells and mesenchymal progenitor cells form blood vessels and increase blood flow in ischemic muscle. *Sci Rep-uk* 7, 770 (2017).

18. Chen, Y. *et al.* Functional Human Vascular Network Generated in Photocrosslinkable Gelatin Methacrylate Hydrogels. *Adv Funct Mater* 22, 2027–2039 (2012).

19. Lin, R.-Z. *et al.* Host non-inflammatory neutrophils mediate the engraftment of bioengineered vascular networks. *Nat Biomed Eng* 1, 0081 (2017).

20. Lin, R.-Z. *et al.* Human endothelial colony-forming cells serve as trophic mediators for mesenchymal stem cell engraftment via paracrine signaling. *Proc National Acad Sci* 111, 10137–10142 (2014).

21. Silvestre-Roig, C., Braster, Q., Ortega-Gomez, A. & Soehnlein, O. Neutrophils as regulators of cardiovascular inflammation. *Nat Rev Cardiol* 17, 327–340 (2020).

22. Horckmans, M. *et al.* Neutrophils orchestrate post-myocardial infarction healing by polarizing macrophages towards a reparative phenotype. *Eur Heart J* 38, 187–197 (2017).

23. Yang, W. *et al.* Neutrophils promote the development of reparative macrophages mediated by ROS to orchestrate liver repair. *Nat Commun* 10, 1076 (2019).

24. Ma, Y. *et al.* Temporal neutrophil polarization following myocardial infarction. *Cardiovasc Res* 110, 51–61 (2016).

25. Leden, R. E. von, Parker, K. N., Bates, A. A., Noble-Haeusslein, L. J. & Donovan, M. H. The emerging role of neutrophils as modifiers of recovery after traumatic injury to the developing brain. *Exp Neurol* 317, 144–154 (2019).

26. Kolaczkowska, E. & Kubes, P. Neutrophil recruitment and function in health and inflammation. *Nat Rev Immunol* 13, 159–175 (2013).

27. Blázquez-Prieto, J., López-Alonso, I., Huidobro, C. & Albaiceta, G. M. The Emerging Role of Neutrophils in Repair after Acute Lung Injury. *Am J Resp Cell Mol* 59, 289–294 (2018).

28. Ohms, M., Möller, S. & Laskay, T. An Attempt to Polarize Human Neutrophils Toward N1 and N2 Phenotypes in vitro. *Front Immunol* 11, 532 (2020).

29. Voskuhl, R. A new cell subtype that confers neuroprotection. *Nat Immunol* 21, 1477–1478 (2020).

30. Sas, A. R. *et al.* A new neutrophil subset promotes CNS neuron survival and axon regeneration. *Nat Immunol* 21, 1496–1505 (2020).

31. Hou, Y. *et al.* N2 neutrophils may participate in spontaneous recovery after transient cerebral ischemia by inhibiting ischemic neuron injury in rats. *Int Immunopharmacol* 77, 105970 (2019).

32. Cochain, C. *et al.* The Chemokine Decoy Receptor D6 Prevents Excessive Inflammation and Adverse Ventricular Remodeling After Myocardial Infarction. *Arteriosclerosis Thrombosis Vasc Biology* 32, 2206–2213 (2012).

33. Vafadarnejad, E. *et al.* Dynamics of Cardiac Neutrophil Diversity in Murine Myocardial Infarction. *Circ Res* 127, e232–e249 (2020).

34. Andreadou, I. *et al.* Immune cells as targets for cardioprotection: new players and novel therapeutic opportunities. *Cardiovasc Res* 115, 1117–1130 (2019).

35. Calcagno, D. M. *et al.* SiglecF(HI) Marks Late-Stage Neutrophils of the Infarcted Heart: A Single-Cell Transcriptomic Analysis of Neutrophil Diversification. *J Am Hear Assoc Cardiovasc Cerebrovasc Dis* 10, e019019 (2021).

36. Lin, R.-Z. & Melero-Martin, J. M. Fibroblast growth factor-2 facilitates rapid anastomosis formation between bioengineered human vascular networks and living vasculature. *Methods* 56, 440–451 (2012).

37. Melero-Martin, J. M. *et al.* Engineering Robust and Functional Vascular Networks In Vivo With Human Adult and Cord Blood–Derived Progenitor Cells. *Circ Res* 103, 194–202 (2008).

38. Li, J. *et al.* All Roads Lead to Rome (the Heart): Cell Retention and Outcomes From Various Delivery Routes of Cell Therapy Products to the Heart. *J Am Hear Assoc Cardiovasc Cerebrovasc Dis* 10, e020402.

39. Zhao, R., Su, Z., Wu, J. & Ji, H.-L. Serious adverse events of cell therapy for respiratory diseases: a systematic review and meta-analysis. *Oncotarget* 8, 30511–30523 (2017).

40. Liu, Z. *et al.* Systemic and local delivery of mesenchymal stem cells for heart renovation: Challenges and innovations. *Eur J Pharmacol* 876, 173049 (2020).

41. Kindi, A. A., Ge, Y., Shum-Tim, D. & Chiu, R. C.-J. Cellular cardiomyoplasty: routes of cell delivery and retention. *Front Biosci* 13, 2421 (2008).

42. Vrtovec, B. *et al.* Comparison of Transendocardial and Intracoronary CD34+ Cell Transplantation in Patients With Nonischemic Dilated Cardiomyopathy. *Circulation* 128, S42–S49 (2013).

43. Hou, D. *et al.* Radiolabeled Cell Distribution After Intramyocardial, Intracoronary, and Interstitial Retrograde Coronary Venous Delivery. *Circulation* 112, I150-6 (2005).

44. Akker, F. van den *et al.* Intramyocardial stem cell injection: go(ne) with the flow. *Eur Heart J* 38, 184–186 (2017).

45. Park, S.-J. *et al.* Dual stem cell therapy synergistically improves cardiac function and vascular regeneration following myocardial infarction. *Nat Commun* 10, 3123 (2019).

46. Teng, C. J., Luo, J., Chiu, R. C. J. & Shum-Tim, D. Massive mechanical loss of microspheres with direct intramyocardial injection in the beating heart: Implications for cellular cardiomyoplasty. *J Thorac Cardiovasc Surg* 132, 628–632 (2006).

47. Abdelwahid, E. *et al.* Stem cell death and survival in heart regeneration and repair. *Apoptosis* 21, 252–268 (2016).

48. Li, Z., Hu, S. & Cheng, K. Chemical Engineering of Cell Therapy for Heart Diseases. *Accounts Chem Res* 52, 1687–1696 (2019).

49. Huang, K., Hu, S. & Cheng, K. A New Era of Cardiac Cell Therapy: Opportunities and Challenges. *Adv Healthc Mater* 8, 1801011 (2019).

50. Garcia, J. R. *et al.* A Minimally Invasive, Translational Method to Deliver Hydrogels to the Heart Through the Pericardial Space. *Jacc Basic Transl Sci* 2, 601–609 (2017).

51. Tang, J. *et al.* Heart Repair Using Nanogel-Encapsulated Human Cardiac Stem Cells in Mice and Pigs with Myocardial Infarction. *Acs Nano* 11, 9738–9749 (2017).

52. Cui, X. *et al.* NIPAM-based Microgel Microenvironment Regulates the Therapeutic Function of Cardiac Stromal Cells. *Acs Appl Mater Inter* 10, 37783–37796 (2018).

53. Ichihara, Y. *et al.* Self-assembling peptide hydrogel enables instant epicardial coating of the heart with mesenchymal stromal cells for the treatment of heart failure. *Biomaterials* 154, 12–23 (2018).

54. Cheng, K. *et al.* Functional performance of human cardiosphere-derived cells delivered in an in situ polymerizable hyaluronan-gelatin hydrogel. *Biomaterials* 33, 5317–5324 (2012).

55. Chuang, C.-H., Lin, R.-Z., Melero-Martin, J. M. & Chen, Y.-C. Comparison of covalently and physically cross-linked collagen hydrogels on mediating vascular network formation for engineering adipose tissue. *Artif Cells Nanomed Biotechnology* 46, 1–14 (2018).

56. Kuo, K.-C. *et al.* Bioengineering vascularized tissue constructs using an injectable cell-laden enzymatically crosslinked collagen hydrogel derived from dermal extracellular matrix. *Acta Biomater* 27, 151–166 (2015).

57. Chuang, C.-H. *et al.* Enzymatic regulation of functional vascular networks using gelatin hydrogels. *Acta Biomater* 19, 85–99 (2015).

58. Nichol, J. W. *et al.* Cell-laden microengineered gelatin methacrylate hydrogels. *Biomaterials* 31, 5536–5544 (2010).

59. Sharifi, S., Sharifi, H., Akbari, A. & Chodosh, J. Systematic optimization of visible light-induced crosslinking conditions of gelatin methacryloyl (GelMA). *Sci Rep-uk* 11, 23276 (2021).

60. Noshadi, I. *et al.* In vitro and in vivo analysis of visible light crosslinkable gelatin methacryloyl (GelMA) hydrogels. *Biomater Sci-uk* 5, 2093–2105 (2017).

61. Roche, E. T. *et al.* A light-reflecting balloon catheter for atraumatic tissue defect repair. *Sci Transl Med* 7, 306ra149 (2015).

62. Ptaszek, L. M. *et al.* Gelatin Methacryloyl Bioadhesive Improves Survival and Reduces Scar Burden in a Mouse Model of Myocardial Infarction. *J Am Hear Assoc Cardiovasc Cerebrovasc Dis* 9, e014199 (2020).

Figure 1

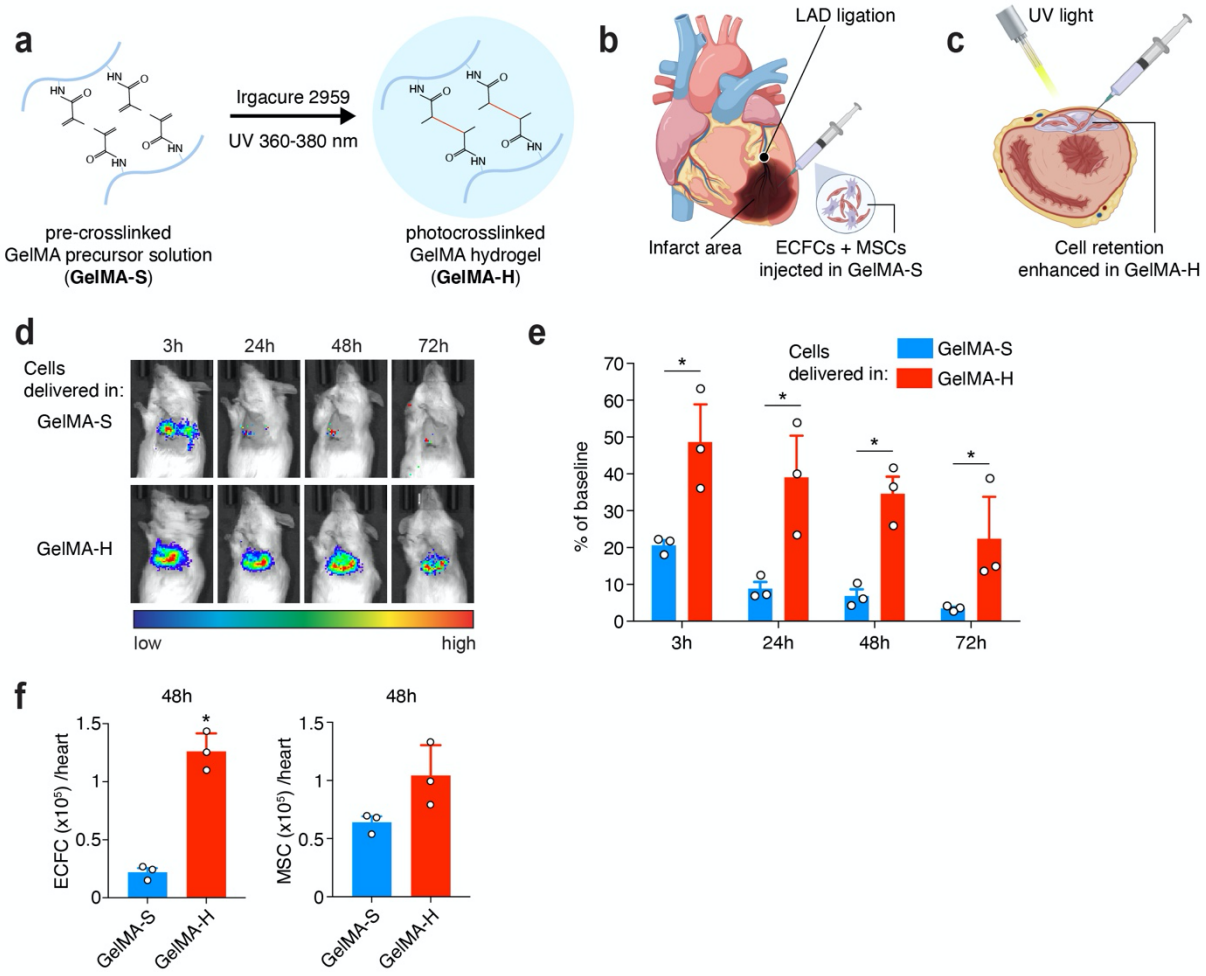

Figure 1. Enhanced intramyocardial cell retention by delivering human vascular progenitor cells in photocrosslinked GelMA hydrogel. (A) Schematic photocrosslinking of GelMA from a precursor solution (GelMA-S) to a hydrogel matrix (GelMA-H) under UV irradiation. Free radicals generated by the photoinitiator 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 precursor solution were injected into three locations in the infarct area. (C) UV light was applied on top of the infarcted area to polymerize a cell-laden hydrogel (referred to as GelMA-H group). UV irradiation was omitted for the control group to maintain cells injected in GelMA precursor 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 on 3, 24, 48, and 72 h post-MI and compared to the baseline (measured by cells delivered to arrested mouse hearts). (F) Retention of human ECFCs or MSCs was quantified by flow cytometric analysis on 48 h. Data were displayed by counting cell numbers from each harvested MI heart. Data are mean ±s.d.; n = 3 mice per group (indicated by individual dots). *P < 0.005 between GelMA-H and GelMA-S. Statistical methods: two-way ANOVA followed by Bonferroni's post-test analysis (E) and unpaired two-tailed Student's t-tests (F).

Figure 2

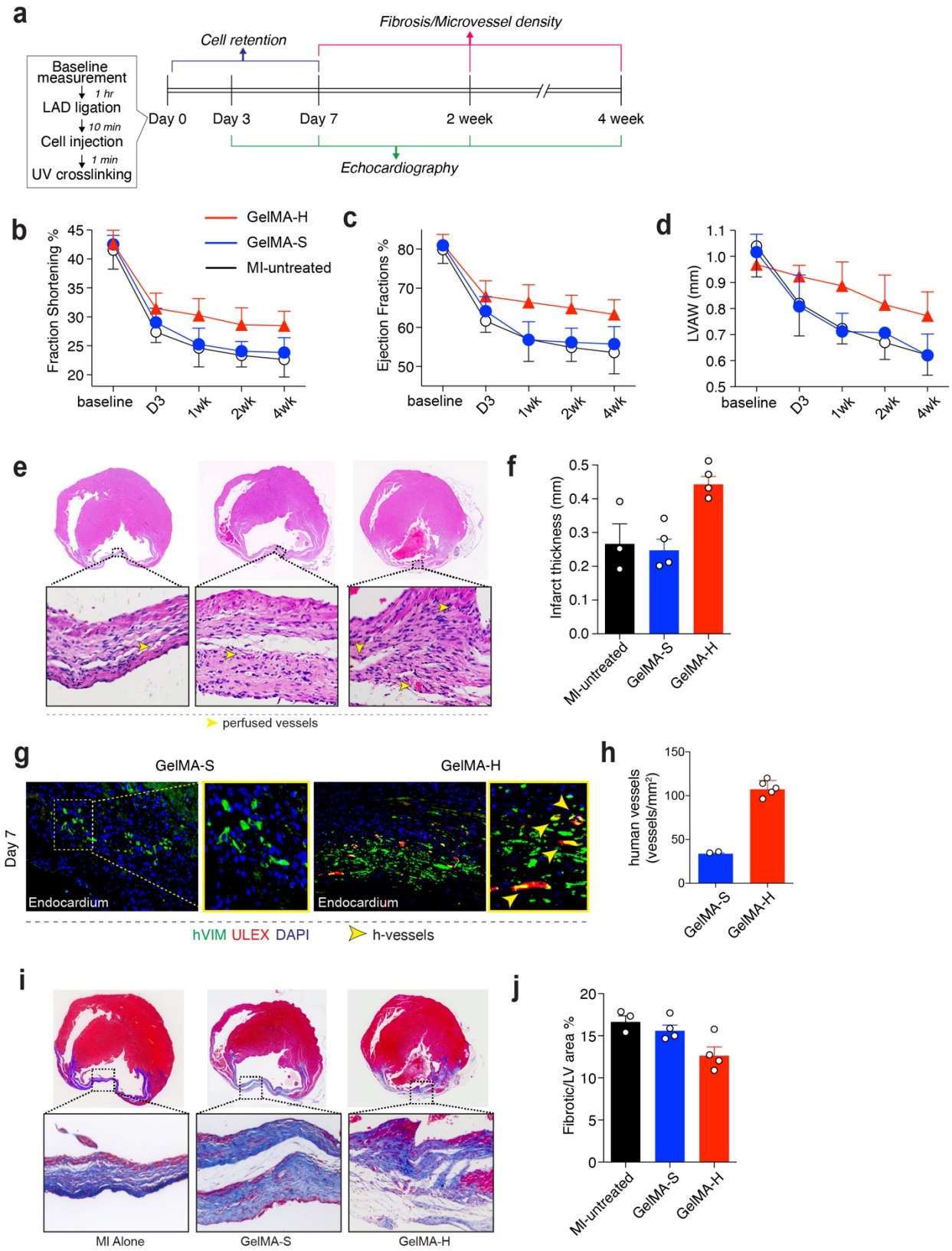

Figure 2. Human vascular cells delivered in GelMA hydrogel promoted beneficial cardiac remodeling. (A) Diagram of experimental schedule. (B-D) Echocardiography analysis for cardiac function. Echocardiography was performed on anesthetized mice at 3 days, and 1, 2, 4 weeks

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 control (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) 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 were identified by immunostaining for human-specific vimentin (hVIM) and UEA-1 lectin binding. Scale bars, xxx mm. (H) Total perfused human microvessel density quantified in infarct myocardium. (I) Representative heart sections stained with Masson's trichrome. Scale bars, xxx mm. (J) The percentage of fibrotic areas in the total LV cross-sections quantified by Masson's trichrome staining. 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.

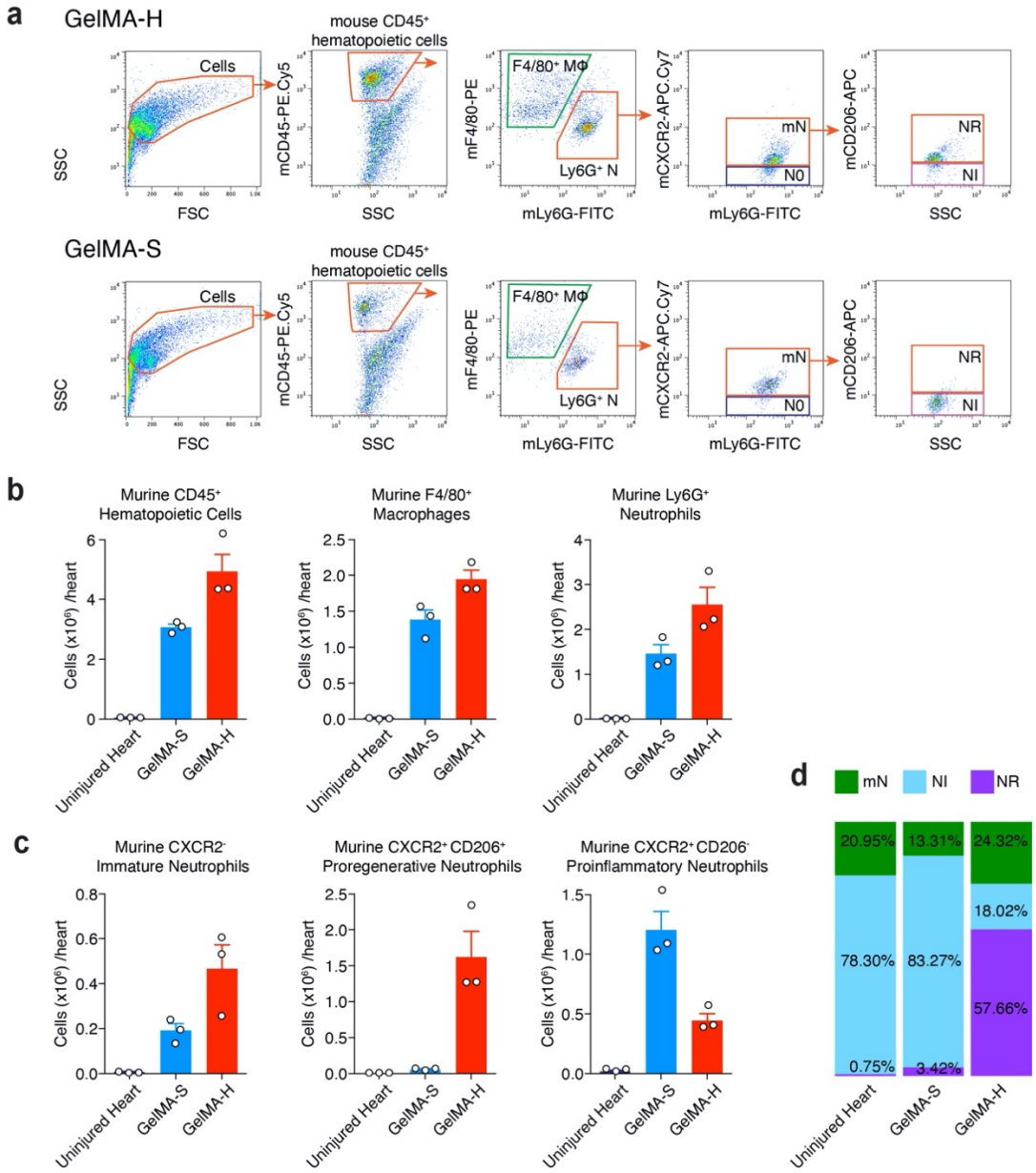

Figure 3. Recruitment of neutrophils with reparative phenotype in GelMA-H group. Infarcted myocardium was harvested on day 2 post-MI. Tissues were digested into single-celled suspension for flow cytometric analysis. (A) The flow cytometric gating strategy to profile recruited myeloid cells. Representative flow cytometry analyses of ischemic myocardium injected with ECFC+MSC in GelMA-S or GelMA-H. MΦ, macrophages; N, neutrophils; mN, mature neutrophils; N0, naïve neutrophils; NR, pro-regenerative neutrophils; NI, pro-inflammatory neutrophils. (B) Quantitative cytometric analyses of murine CD45+ hematopoietic cells, macrophages, and total neutrophils obtained from ischemic myocardium (n=3; means $\pm$ SD). The uninjured hearts served as controls. (C) Recruited $Ly6G^{+}F4/80^{-}$ neutrophils were classified into three subpopulations: N0 ($CXCR2^{-}$), NI ($CXCR2^{+}CD206^{-}$), and NR ($CXCR2^{+}CD206^{+}$). (D) Proportions of the three neutrophil subpopulations in each group.

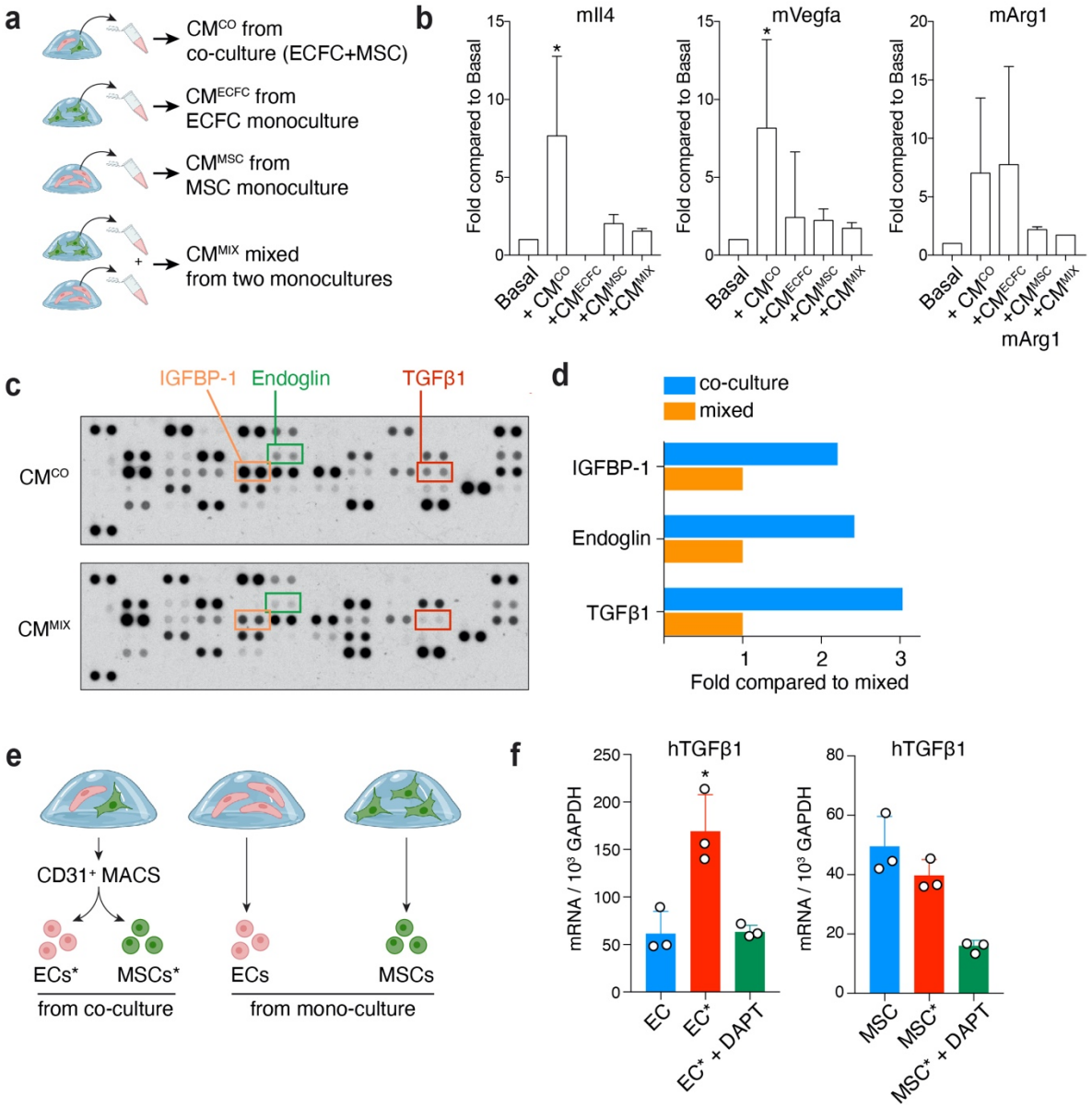

Figure 4. Human vascular cell interaction upregulated NR polarization factor TGFb1. (A) Conditioned media (CM) were collected from ECFC+MSC coculture in GelMA hydrogel over 7 days in vitro (CM^{CO}). CMs obtained from ECFC or MSC monoculture (CM^{ECFC} or CM^{MSC}) were mixed in 1:1 ratio (termed CM^{MIX}) served as a control. The secretions of cytokines were measured by proteomic profiler arrays. Selected cytokines are marked with colored outlines. (B) Quantification of cytokine levels was carried out by densitometry. (C) ECFC+MSC were cocultured in GelMA hydrogel and separated by MACS for gene expression analyses. ECFC or MSC monoculture served as controls. (D) Quantitative PCR analyses of TGFβ1 expression in ECFCs sorted from ECFC+MSC coculture (labeled EC*) or from ECFC monoculture (EC). The same labeling applied to MSC (monoculture) and MSC* (sorted from coculture). DAPT was added to ECFC+MSC coculture to block Notch-mediated perivascular interaction. Data present mean ± SD. The numbers above bars are P values.

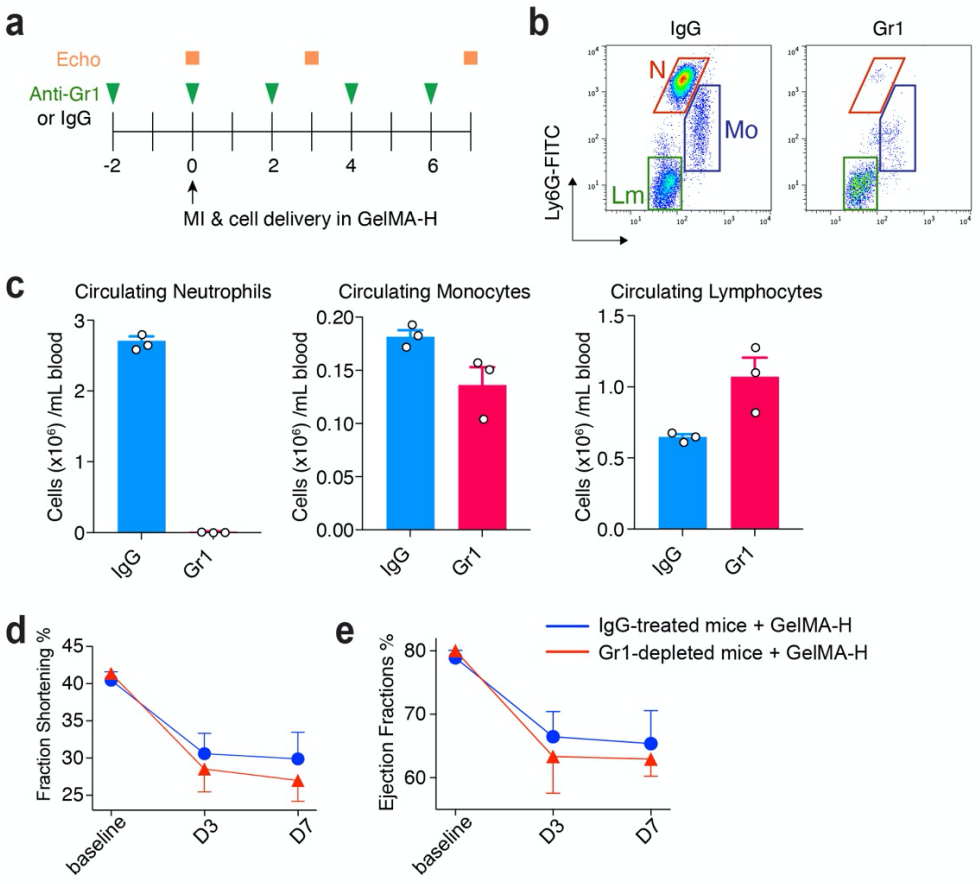

Figure 5. Neutrophil depletion canceled the benefit of human vascular cell delivery. (A) Experimental schedule: Anti-Gr-1 or IgG control antibody (200 µg each) was given by intraperitoneal injection every 2 days. ECFC+MSC were delivered in GelMA-H into the ischemic myocardium at day 0. Cardiac functions were measured by echocardiography within 1h before performing LAD ligation to obtain the baseline data for each animal. The effect of anti-Gr-1 depletion was monitored on days 3 and 7 post-MI. (B) Representative flow cytometry analyses of circulating leukocytes from the blood specimens of anti-Gr-1-treated or IgG-treated mice on day 2. (C) Quantitative cytometric analyses of circulating leukocytes were obtained from anti-Gr-1-treated or IgG-treated mice (n=3; means ± SD). (D) Left ventricular fractional shortening (LVFS). (E) Left ventricular ejection fraction (LVEF). Statistical methods: unpaired two-tailed Student's t-tests (C) and ANOVA followed by Bonferroni's post-test analysis (D). Data present mean ± SD. The numbers above bars are P values.