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Controlled Inhibition of the Mesenchymal Stromal Cell Pro-inflammatory Secretome via Microparticle Engineering

Sudhir H. Ranganath,^{1,2,3,4,6} Zhixiang Tong,^{2,4,5} Oren Levy,^{2,4,5} Keir Martyn,^{2,4,5} Jeffrey M. Karp,^{2,4,5,*} and Maneesha S. Inamdar^{1,3,*}

¹Molecular Biology and Genetics Unit, Jawaharlal Nehru Center for Advanced Scientific Research, Jakkur, Bangalore 560064, India

²Division of Biomedical Engineering, Department of Medicine, Brigham & Women's Hospital, Harvard Medical School, Cambridge, MA 02139, USA

³Institute for Stem Cell Biology and Regenerative Medicine, GKVK - Post, Bellary Road, Bangalore 560065, India

⁴Harvard-MIT Division of Health Sciences and Technology, 65 Landsdowne Street, Cambridge, MA 02139, USA

⁵Harvard Stem Cell Institute, 1350 Massachusetts Avenue, Cambridge, MA 02138, USA

⁶Department of Chemical Engineering, Siddaganga Institute of Technology, B.H. Road, Tumkur 572103, India

*Correspondence: jeffkarp.bwh@gmail.com (J.M.K.), inamdar@jncasr.ac.in (M.S.I.)

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SUMMARY

Mesenchymal stromal cells (MSCs) are promising therapeutic candidates given their potent immunomodulatory and anti-inflammatory secretome. However, controlling the MSC secretome post-transplantation is considered a major challenge that hinders their clinical efficacy. To address this, we used a microparticle-based engineering approach to non-genetically modulate pro-inflammatory pathways in human MSCs (hMSCs) under simulated inflammatory conditions. Here we show that microparticles loaded with TPCA-1, a small-molecule NF- κ B inhibitor, when delivered to hMSCs can attenuate secretion of pro-inflammatory factors for at least 6 days in vitro. Conditioned medium (CM) derived from TPCA-1-loaded hMSCs also showed reduced ability to attract human monocytes and prevented differentiation of human cardiac fibroblasts to myofibroblasts, compared with CM from untreated or TPCA-1-preconditioned hMSCs. Thus, we provide a broadly applicable bioengineering solution to facilitate intracellular sustained release of agents that modulate signaling. We propose that this approach could be harnessed to improve control over MSC secretome post-transplantation, especially to prevent adverse remodeling post-myocardial infarction.

INTRODUCTION

Mesenchymal stromal cells (MSCs; also known as bone marrow stromal cells and earlier known as mesenchymal stem cells) are being explored as therapeutics in over 550 clinical trials registered with the US Food and Drug Administration (www.FDA.gov) for the treatment of a wide range of diseases (Ankrum et al., 2014c). Their immunoevasive properties (Ankrum et al., 2014c) and safe transplant record, allowing allogeneic administration without an immunosuppressive regimen, positions MSCs as an appealing candidate for a potential off-the-shelf product. One of the primary mechanisms exploited in MSC therapeutics is a secretome-based paracrine effect as evidenced in many pre-clinical studies (Ranganath et al., 2012). However, controlling the MSC secretome post-transplantation is considered a major challenge that hinders their clinical efficacy. For instance, upon transplantation, MSCs are subjected to a complex inflammatory milieu (soluble mediators and immune cells) in most injury settings. MSCs not only secrete anti-inflammatory factors, but also produce pro-inflammatory factors that may compromise their therapeutic efficacy. Table S1 lists a few in vitro and in vivo conditions that demonstrate the complex microenvironment under which MSCs switch between anti-inflammatory and pro-inflammatory phenotypes.

Levels of pro- or anti-inflammatory cytokines are not always predictive of the response, possibly due to the

dynamic cytokine combinations (and concentrations) present in the cell microenvironment (Table S1). For example, relatively low inflammatory stimulus (<20 ng/ml tumor necrosis factor alpha [TNF- α] alone or along with interferon- γ [IFN- γ]) can polarize MSCs toward pro-inflammatory effects (Bernardo and Fibbe, 2013) resulting in increased inflammation characterized by T cell proliferation and transplant rejection. Conversely, exposure to high levels of the inflammatory cytokine TNF- α has been shown in certain studies to result in MSC-mediated anti-inflammatory effects via secretion of potent mediators such as TSG6, PGE2, STC-1, IL-1Ra, and sTNFR1 as demonstrated in multiple inflammation-associated disease models (Prockop and Oh, 2012; Ylostalo et al., 2012). These effects are mediated via molecular pathways such as NF- κ B, PI3K, Akt, and JAK-STAT (Ranganath et al., 2012). However, it is not clear that low and high levels of TNF- α always exert the same effect on anti- versus pro-inflammatory MSC secretome. NF- κ B is a central regulator of the anti-inflammatory secretome response in monolayer (Yagi et al., 2010), spheroid MSCs (Bartosh et al., 2013; Ylostalo et al., 2012) and TNF- α -mediated (20 ng/ml for 120 min) apoptosis (Peng et al., 2011). Given that NF- κ B can promote secretion of pro-inflammatory components in the MSC secretome (Lee et al., 2010), we hypothesized that NF- κ B inhibition via small molecules in MSC subjected to a representative inflammatory stimulus (10 ng/ml TNF- α) would inhibit their pro-inflammatory responses.



Adverse remodeling or cardiac fibrosis due to differentiation of cardiac fibroblasts (CF) into cardiac myofibroblasts (CMF) with pro-inflammatory phenotype and collagen deposition is the leading cause for heart failure. The secretome from exogenous MSCs has anti-fibrotic and angiogenic effects that can reduce scar formation (Preda et al., 2014) and improve ejection fraction when administered early or prior to adverse remodeling (Preda et al., 2014; Tang et al., 2010; Williams et al., 2013). Unfortunately in many cases, due to poor prognosis, MSCs may not be administered in time to prevent adverse remodeling to inhibit CF differentiation to CMF (Virag and Murry, 2003) or to prevent myocardial expression of TNF- α (Bozkurt et al., 1998; Mann, 2001). Also, when administered following an adverse remodeling event including CMF differentiation, MSCs may assume a pro-inflammatory phenotype and secretome (Naftali-Shani et al., 2013) under TNF- α (typically \sim 5 pg/mg of total protein in myocardial infarction (MI) rat myocardium which is not significantly higher than 1–3 pg/mg protein in control rat myocardium) (Moro et al., 2007) resulting in impaired heart function. Hence, if the pro-inflammatory response of hMSCs can be suppressed it may maximize efficacy.

While the MSC phenotype can be controlled under regulated conditions in vitro, the in vivo response of MSCs post-transplantation is poorly controlled as it is dictated by highly dynamic and complex host microenvironments (Discher et al., 2009; Rodrigues et al., 2010). Factors including MSC tissue source (Melief et al., 2013; Naftali-Shani et al., 2013), donor and batch-to-batch variability with respect to cytokine secretion and response to inflammatory stimuli (Zhukareva et al., 2010), gender (Crisostomo et al., 2007), and age (Liang et al., 2013) also affect the response of MSCs. Thus, it is important to develop approaches to control the MSC secretome post-transplantation regardless of their source or expansion conditions. We hypothesized that engineering MSCs to induce a specific secretome profile under a simulated host microenvironment may maximize their therapeutic utility. Previously, the MSC secretome has been regulated via genetic engineering (Gnecchi et al., 2006; Wang et al., 2009) or cytokine/small-molecule preconditioning approaches (Crisostomo et al., 2008; Mias et al., 2008). Genetically engineered human MSCs (hMSCs) pose challenging long-term regulatory hurdles given that the potential tumorigenicity has not been well characterized, and while preconditioning hMSCs with cytokines/small molecules may be safer, the phenotype-altering effects are transient.

In this study, we aimed to modulate the hMSC secretome with a small molecule, specifically inhibiting the pro-inflammatory components while maintaining few anti-inflammatory and angiogenic components. Through this strategy, we sought to minimize CMF differentiation and

inhibit total collagen deposition, which are primary mediators of cardiac fibrosis. Using TNF- α stimulated NF- κ B activation as a model of inflammation, we show that microparticle-mediated continuous delivery of an NF- κ B inhibitor can provide sustained inhibition of the hMSC pro-inflammatory secretome and thereby inhibit monocyte migration, CMF differentiation, and collagen deposition.

RESULTS

Profiling the hMSC Secretome upon TNF- α Stimulation

To identify relevant targets for inhibition of the pro-inflammatory secretome, we profiled hMSCs treated with the pro-inflammatory stimulus TNF- α by quantifying over 250 cytokines and chemokines in the hMSC conditioned medium (CM) using antibody arrays (Figure 1A). See Table 1 for details of the experimental groups. Upon TNF- α stimulation, there was notable increase in the secretion of multiple cytokines and chemokines in the CM compared with control-hMSCs (Figure 1B). While constitutively secreted pro-inflammatory cytokines IL-6, IL-8, MCP-1, and MCP-3 showed modest increase (\sim 19-, \sim 5-, \sim 2.6-, and \sim 4-fold, respectively), TNF- α stimulation resulted in secretion of pro-inflammatory cytokines RANTES (CCL5), ENA78, GRO, and GCP-2 at significantly elevated concentrations (\sim 4,000-, \sim 133-, \sim 17-, and \sim 63-fold, respectively) compared with control-hMSCs (Figure 1B).

TPCA-1 Inhibits Secretion of Pro-inflammatory Cytokines from TNF- α -Stimulated hMSCs

To investigate whether the increased secretion of pro-inflammatory factors upon TNF- α stimulation in hMSCs is mediated by NF- κ B, we used small-molecule inhibitors of NF- κ B, namely PSI (proteasome inhibitor) and TPCA-1 (IKK β inhibitor). PSI was highly toxic to the cells (IC₅₀ \sim 3 μ M; where IC₅₀ is the median inhibitory concentration) when treated for 48 hr (Figure S1A), but at lower concentration (10 nM) PSI did not inhibit secretion of IL-6, a pro-inflammatory cytokine secreted at high levels upon TNF- α stimulation of hMSCs (Figure S1B). In contrast, TPCA-1 had low toxicity on hMSCs (IC₅₀ \sim 90 μ M) when treated for 48 hr (Figure S1C) and 10 μ M concentration was chosen for future experiments. In addition, TPCA-1 treatment at 10 μ M did not inhibit osteogenic and adipogenic differentiation of hMSCs (Figures S2A and S2B).

To test the utility of TPCA-1 as a reversible modulator of the NF- κ B mediated pro-inflammatory secretome, we tested its effect on cytokine production by TNF- α activated hMSCs. Control (control-hMSC), TNF- α -treated (TNF-hMSC), and TNF- α /TPCA-1-treated hMSC (TNF + TPCA-hMSC) were

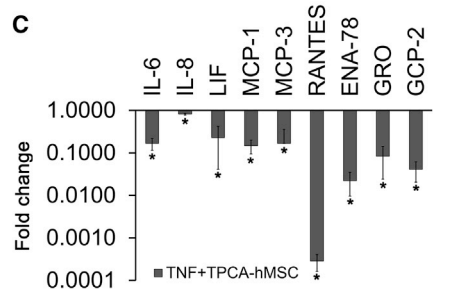
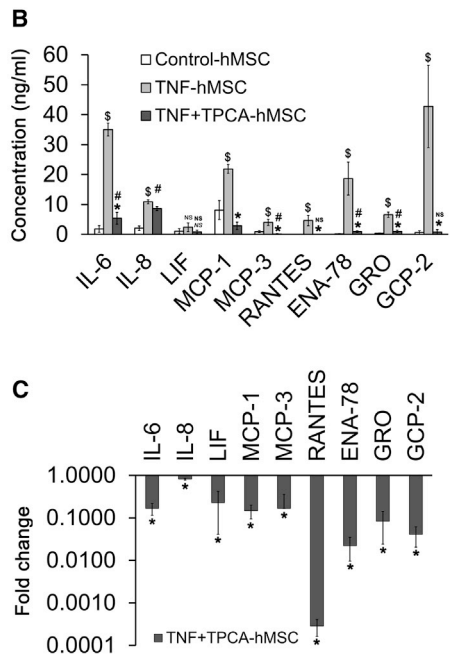
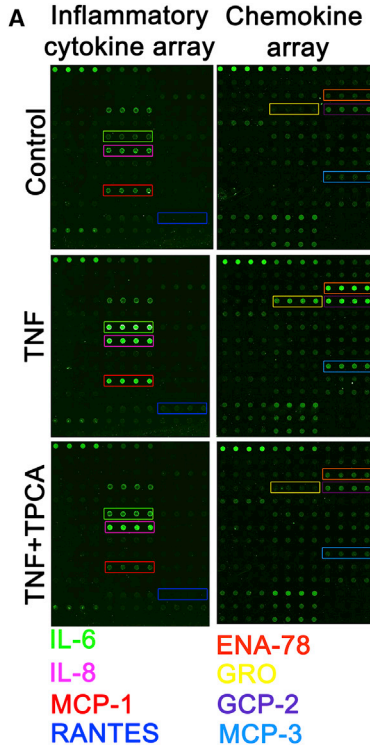


Figure 1. Characterization of hMSC Pro-inflammatory Secretome upon TNF- α Stimulation and TPCA-1 Treatment

(A) Fluorescence images of cytokine antibody arrays obtained via laser scanning microplate reader from multiple hMSC secretome samples.

(B) Concentration profile of hMSC secretome obtained via quantitative analysis of RayBiotech Quantibody arrays using GenePix Pro software. Of 250 cytokines, chemokines, and growth factors profiled, mediators that were statistically significant among the three hMSC samples are reported. @ refers to 1/10th of the actual concentration of ENA78. \$ and NS indicate $p < 0.01$ and not significant between secretome from control-hMSC and TNF-hMSC, respectively; * and NS indicate $p < 0.01$ and not significant between secretome from TNF-hMSC and TNF + TPCA-hMSC, respectively; # and NS indicate $p < 0.01$ and not significant between secretome from control-hMSC and TNF + TPCA-hMSC, respectively.

(C) Fold change in concentration of hMSC secretome components normalized to concentration of secretome components in TNF-

hMSC. p values indicate significance ($*p < 0.01$) between secretome from TNF-hMSC and TNF + TPCA-hMSC. Data represent the average of duplicates from three independent experiments from two donor MSCs. Error bars represent \pm SD.

incubated for 48 hr in serum-free medium. Sustained TPCA-1 treatment in TNF + TPCA-hMSCs significantly reduced the secreted levels of pro-inflammatory mediators MCP-1, RANTES, GCP-2, LIF, and MCP-3 to lower than or equivalent to the basal levels of control-hMSCs (Figure 1B). Secreted levels of IL-6, IL-8, and GRO were significantly lower than levels secreted by TNF-hMSCs. In addition, reduction in cytokine secretion levels for TNF + TPCA-hMSC CM versus TNF-hMSC CM were as follows: IL-6 (~6-fold), IL-8 (~1.2-fold), LIF (~9-fold), MCP-1 (~7-fold), MCP-3 (~3-fold), RANTES (~5,400-fold), ENA78 (~45-fold), GRO (~6-fold), and GCP-2 (~58-fold) (Figure 1C).

Next, we sought to examine if TPCA-1 would inhibit the beneficial paracrine effect of hMSCs mediated by several pathways including JAK-STAT, PI3/Akt, and MAPK (Ranganath et al., 2012). Concentrations of growth factors, cytokines, and anti-inflammatory mediators were quantified from Quantibody arrays. Secretion of most growth factors (Figures S3A and S3B) and cytokines (Figures S3C and S3D) was unaffected even upon TNF- α stimulation and TPCA-1 treatment, except for HGF-1 the secretion of which was significantly inhibited upon TPCA-1 treatment (Figure S3A). However, secretion of platelet-derived growth factor-AA and anti-inflammatory cytokine TSG-6 was significantly augmented upon TNF- α stimulation and

maintained at elevated levels even upon TPCA-1 treatment (Figures S3A and S3D).

Functionality of the inhibited pro-inflammatory hMSC secretome was characterized by investigating transwell migration of human monocytes in vitro (Figure S4A). Figure S4B indicates that TPCA-1 treatment significantly inhibited transwell migration of human monocytes in response to TNF-hMSC secretome. This, along with the inhibition of pro-inflammatory secretome by TPCA-1 prompted us to develop a non-genetic, autonomous controlled release approach for modulating pro-inflammatory secretome in hMSCs.

Surface Modified PLGA Microparticles Delivering TPCA-1 are Internalized into hMSCs

Previously, we demonstrated a particle-in-cell approach where 1–2 μ m drug-loaded poly(D,L-lactide-co-glycolide) (PLGA) microparticles were internalized into hMSCs and retained at least for 7 days and potentially weeks (Sarkar et al., 2011). Drugs released from these particle-engineered hMSCs demonstrated sustained phenotype-altering effects (Ankrum et al., 2014a; Sarkar et al., 2011). In this study, we developed 1–2 μ m PLGA microparticles encapsulating TPCA-1 using the single-emulsion solvent evaporation technique. PLGA with a glycolic acid to lactic acid ratio of 50:50 and



Table 1. Abbreviations and Expanded Forms of the Experimental Groups

Abbreviations	Description of the Experimental Groups
Control-hMSC	treated with 1% DMSO for 48 hr
TNF-hMSC	treated with 10 ng/ml TNF- α and 1% DMSO for 48 hr
TNF + TPCA-hMSC	treated with 10 ng/ml TNF- α and 10 μ M TPCA-1 for 48 hr
TPCA-hMSC	treated with 10 μ M TPCA-1 for 48 hr
TPCApre + TNF-hMSC	pre-treated with 10 μ M TPCA-1 for 24 hr and after removal of TPCA-1, treated with 10 ng/ml TNF- α every 48 hr
TPCApre-hMSC	pre-treated with 10 μ M TPCA-1 for 24 hr
μ P-hMSC	loaded with 200 μ g/ml blank microparticles and incubated for 24 hr
TNF + μ P-hMSC	loaded with 200 μ g/ml blank microparticles, incubated for 24 hr and treated with 10 ng/ml TNF- α every 48 hr
TPCA μ P-hMSC	loaded with 200 μ g/ml TPCA-microparticles and incubated for 24 hr
TNF + TPCA μ P-hMSC	loaded with 200 μ g/ml TPCA-microparticles, incubated for 24 hr and treated with 10 ng/ml TNF- α every 48 hr

10 kDa molecular weight was chosen to formulate the microparticles to ensure faster degradation. The microparticles were round in shape with smooth surface morphology (Figure 2A) with a relatively homogeneous size distribution (PDI 0.26) (Figure 2B). Given the anionic surface property of PLGA used in this study, the surface of the microparticles was negatively charged (Figure 2B). Flow cytometry analysis of fluorescent dye-loaded microparticles (D μ Ps) revealed their association with hMSCs (Figure 2C), and confocal imaging confirmed that they were only associated with the cell membrane and not completely internalized (Figure 2D). To ensure internalization, the microparticles were treated with cationic polymer poly-L-lysine (PLL) resulting in reduced negative surface charge (Figure 2B). Flow cytometry analysis confirmed that PLL-coated microparticles had greater interaction with hMSCs (increase in average fluorescent intensity, Figure 2C), and confocal imaging revealed internalization (Figure 2D). The microparticles were formulated with 19% (w/w) TPCA-1 loading and 80% encapsulation efficiency (Figure 2B), and PLL coating did not affect these parameters. The microparticles released TPCA-1 completely within 20 days of incubation in PBS at 37°C with a near zero-order release kinetics (Figure 2E). The microparticles degraded almost completely after 20 days suggesting that the drug was encapsulated. Incubation of hMSCs with TPCA μ P concentrations of up to 200 μ g/ml for 7 days did not affect cell viability (Figure S1D).

TPCA μ Ps Sustainably Inhibit Pro-inflammatory Secretome in TNF- α -Stimulated hMSCs

Next, we compared our microparticle engineering regimen with the conventional preconditioning regimen to investigate if our approach would provide sustained modulation of the hMSC secretome under TNF- α stimulation. From the pro-inflammatory secretome profiled earlier, IL-6, MCP-1 (CCL2), and RANTES (CCL5) were selected for this study as they are known to significantly mediate inflammatory responses in many cell types. Soluble TPCA-1 preconditioning (Figure 3A) could inhibit secretion of RANTES from TNF-hMSC significantly for only 2 days (Figure 3B). Soluble TPCA-1 preconditioning was unable to inhibit IL-6 and MCP-1 secretion in TPCApre + TNF-hMSCs (Figure 3B) throughout the assay. μ P-hMSCs displayed a secretory profile similar to control-hMSCs and, upon TNF- α stimulation, secreted upregulated levels of RANTES, IL-6, and MCP-1 similar to TNF-hMSCs. Notably, TPCA μ P-hMSCs, upon repeated TNF- α stimulation, displayed significant inhibition in secreted levels of RANTES (low to basal level) until 6 days and IL-6 and MCP-1 until 4 days (Figure 3B).

Intracellular TPCA Released from Microparticles Inhibits NF- κ B Nuclear Translocation in TNF- α -Stimulated hMSCs

We then sought to examine the effect of TNF- α and TPCA (exogenous and intracellularly released from microparticles) on NF- κ B signaling in hMSCs. The experimental design is similar to the one depicted in Figure 3A except that the cells were stained 30 min after TNF- α stimulation due to the rapid nuclear translocation of NF- κ B (p65 subunit). The p65 subunit of NF- κ B complex was absent in the nuclei of about 90% of untreated and μ P-hMSCs, whereas stimulation with TNF- α resulted in the translocation of NF- κ B into the nuclei of TNF-hMSCs and TNF + μ P-hMSCs (Figures 3C and 3D). Preconditioning of hMSCs with TPCA also did not inhibit nuclear translocation of NF- κ B suggesting the effect of TPCA is significantly attenuated due to its removal from the media. In contrast, significantly fewer TNF + TPCA μ P-hMSCs (about 20% cells) contained NF- κ B in their nuclei (Figure 3D), suggesting that binding of TPCA to IKK-2 inhibits TNF- α from activating NF- κ B. This suggests that hMSCs could be transiently controlled for response to TNF- α by loading them with TPCA μ Ps.

Secretome from TNF- α -Treated-TPCA μ P-hMSCs Sustainably Inhibits Transwell Migration of Human Monocytic Cell Line and Human Primary CD14⁺ Monocytes

Timely inhibition of monocyte migration is necessary to attenuate irreversible scar formation after MI. To assess

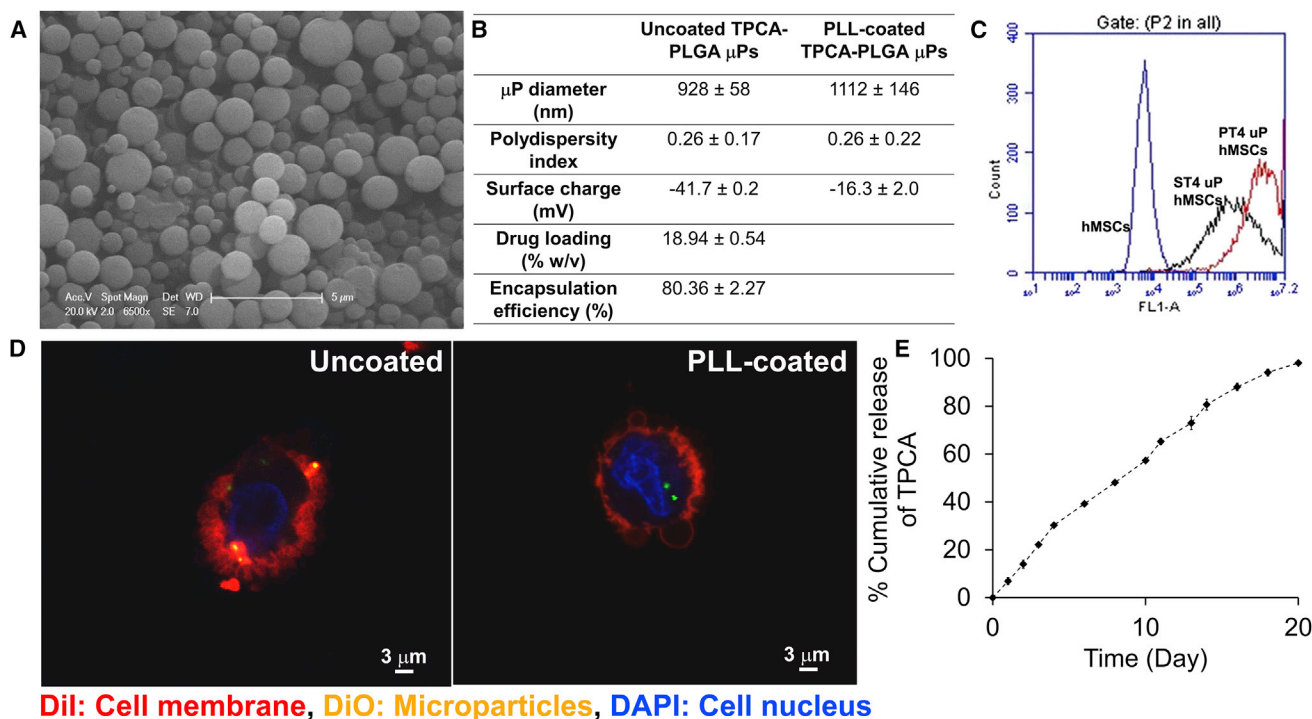


Figure 2. Characterization of TPCA-1-Delivering PLGA Microparticles and Internalization in hMSCs

(A) Representative scanning electron microscope image of TPCA μ Ps showing size, shape, and morphology. (B) Physicochemical properties of TPCA μ Ps. (C) Representative plot of flow cytometry analysis of association of uncoated and PLL-coated D μ Ps in hMSCs. (D) Representative confocal images depicting membrane association of uncoated D μ Ps and cytosolic association of PLL-coated D μ Ps (red, DiI-stained cell membrane; green, D μ Ps; blue, DAPI-stained nucleus). (E) Profile of TPCA release kinetics from 6 mg of TPCA μ Ps into PBS at 37°C. Error bars represent \pm SD.

functionality of the inhibited secretome from TNF + TPCA μ P-hMSCs, we investigated transwell migration of a human monocytic cell line (THP-1) and human primary CD14⁺ monocytes (Figure S4A) in response to hMSC secretome (Figures 4A and 4D). Figures 4B and 4D show that the migration of THP-1 and primary monocytes was dose dependent. This is evident from the large number of cells that migrated toward 100% CM, while 1% CM attracted few cells. CM from days 2, 4, and 6 of TNF-hMSCs augmented migration of THP-1 cells and primary monocytes across the transwell approximately 4- to 8-fold (Figure 4B) and approximately 4-fold (Figure 4D) that of CM from control-hMSCs, respectively. CM from days 2, 4, and 6 of the soluble TPCA preconditioning regimen was ineffective in inhibiting migration of cells across the transwell, even though less significant reduction was observed for day 4 and day 6 CM versus CM from TNF- α -stimulated hMSCs. Conversely, CM from day 2 of TNF + TPCA μ P-hMSCs significantly inhibited migration (3.7-fold and $p < 0.01$) versus TNF-B μ P-hMSCs, and the effect was sustained through day 4 and day 6 although less significantly (2.5-fold, $p < 0.05$ and 1.6-fold, $p < 0.01$, respectively). A 4- to

5-fold reduction was observed in the number of primary monocytes migrated with CM from TNF + TPCA μ P-hMSCs versus CM from TNF-B μ P-hMSCs. Direct comparison of CM from days 2, 4, and 6 reveals that particle-engineered hMSCs are significantly more efficient in reducing migration of monocytes than soluble factor preconditioned hMSCs.

Secretome from TNF- α -Treated-TPCA μ P-hMSCs Inhibits α -SMA Expression and Collagen Production in Human and Murine Cardiac Fibroblasts

CF differentiate into myofibroblasts owing mainly to stimulation by TGF- β , but other pro-inflammatory mediators such as IL-6 and MCP-1 also mediate such pathological differentiation leading to irreversible scar formation upon acute myocardial infarction. Hence we investigated whether the secretome from TNF + TPCA μ P-hMSCs would inhibit α -smooth muscle actin (α -SMA; myofibroblast specific marker) expression and collagen production in CMF. Human (hCF) and mouse (mCF) CF were treated with CM from different hMSC groups for 96 h at 37°C and later immunostained for α -SMA and imaged. hCF receiving day

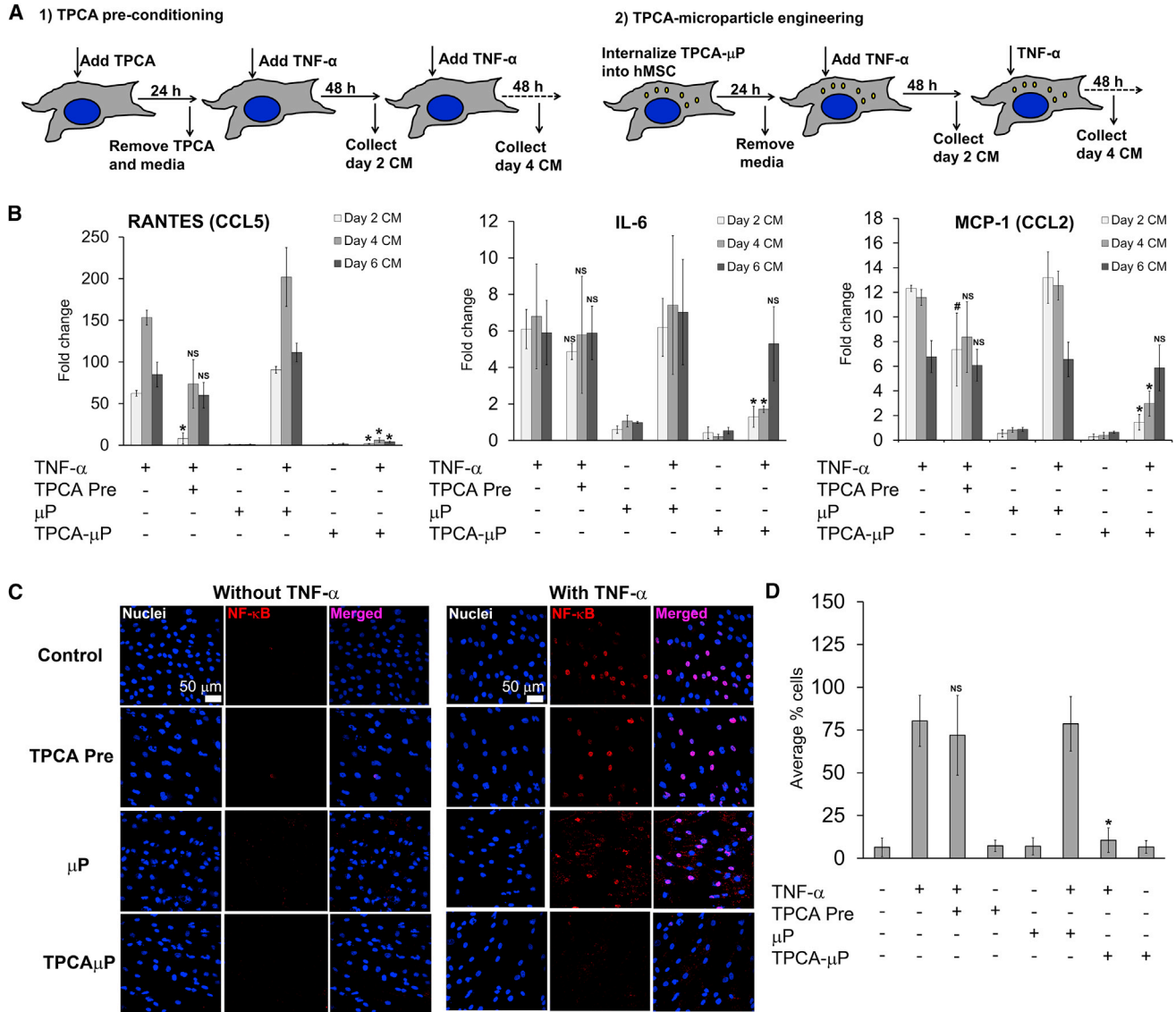


Figure 3. Quantitative Analysis of Secretion of Pro-inflammatory Mediators in hMSC Conditioned Medium and NF- κ B Nuclear Translocation in TPCA Preconditioned and Microparticle-Engineered hMSCs on Days 2, 4, and 6

(A) Experimental design for comparing effects of TPCA preconditioning and microparticle engineering regimens in TNF-hMSC. (B) Fold change in secreted level of RANTES (CCL5), IL-6, and MCP-1 (CCL2) as indicated in hMSCs normalized to the secreted level of control-hMSC. Data represent the average of triplicates from two different hMSC donors. NS represents non-significance versus TNF-hMSC and * indicates $p < 0.01$ versus μ P + TNF-hMSC CM. (C) Representative fluorescence images at 40 \times magnification of hMSCs without microparticles and hMSCs internalized with microparticles after 30 min of TNF- α (10 ng/ml) stimulation. Blue, DAPI (nucleus); red, NF- κ B. (D) Quantitative analysis of percentage of hMSCs positive for nuclear NF- κ B. Each data point is the average of five random fields of view, and data represent the average of triplicates from three independent experiments from two donor hMSCs. NS represents non-significance versus TNF-hMSC, * indicates $p < 0.01$ versus μ P + TNF-hMSC CM, and # represents $p < 0.01$ versus TPCApre + TNF-hMSC CM. Error bars represent \pm SD.

2 CM from control-hMSCs or TNF-hMSCs were significantly positive for α -SMA confirming their CMF phenotype (Figure 5A). CM from TPCApre + TNF-hMSCs could only inhibit α -SMA expression in about 18% of hCF in comparison with

CM from control-hMSCs (Figures 5A and 5B). CM from TNF + μ P-hMSCs had a similar effect on α -SMA expression in hCF in comparison with CM from TNF-hMSCs. However, day 2 CM from TNF + TPCA μ P-hMSC inhibited α -SMA



expression in about 80% of hCF versus CM from control-hMSCs. In addition, CM from particle-engineered hMSCs reduced the α -SMA expression in hCF 4-fold more than CM from preconditioned hMSCs. hMSC media alone or hMSC media with TPCA-1 did not inhibit α -SMA expression (Figure 5B) in hCF. Next, we sought to examine collagen deposition by hMSC CM-treated hCF and mCF using Sirius red staining assay. None of the hMSC CM or hMSC media or hCF media showed detectable collagen levels when assayed alone for Sirius red staining (Figures S5A and S5B). On the other hand, when hCF or mCF were treated in various hMSC media, we could detect significant levels of collagen (Figures 5C, 5D, 5G, and 5H). Within the groups, hCF treated in control-hMSC CM, TNF-hMSC CM, μ P-hMSC CM, and TNF + μ P-hMSC CM groups had significantly higher levels of collagen as quantified by relative staining intensity in Figures 5D and 5H. hCF treated with hMSC media or hMSC media + TPCA-1, secreted collagen at similar levels to control-hMSC CM. Soluble TPCA-1 preconditioned hMSC CM did not significantly inhibit collagen secretion from hCF, but, hCF in TNF + TPCA- μ P-hMSC CM secreted 35% lower amount of collagen compared with control-hMSC CM-treated hCF.

As a first step toward validation in an animal model *in vivo*, we chose to analyze whether TPCA- μ P regulated hMSC secretome would also affect mouse CF differentiation. The effect of hMSC CM on mCF was similar to hCF (Figures 5E and 5F) with respect to α -SMA expression. mCF treated with hMSC media alone or hMSC media with soluble TPCA-1 had elevated levels of α -SMA expression (data not shown) in mCF. However, hMSC CM did not seem to inhibit collagen production of mCF to the extent of hCF (Figures 5G and 5H). More importantly, soluble TPCA-1 preconditioned hMSC CM did not significantly inhibit collagen secretion from mCF. However, compared with CM from TNF + μ P-hMSC, TNF + TPCA- μ P-hMSC CM reduced collagen secretion from mCF by about 24%. This suggests that the inhibited pro-inflammatory secretome from particle-engineered hMSCs attenuate the adoption of fibrotic phenotype changes in both hCF and mCF.

DISCUSSION

The MSC secretome is being explored in pre-clinical and clinical trials as one of the primary mediators of cardiovascular repair via immunomodulatory effects (Ranganath et al., 2012). However, to date, approaches have not met with success as initially hoped. The immunomodulatory property of MSCs presents an unpredictable therapeutic outcome as it is affected by inter-cellular and inter-donor heterogeneity, the MSC source (Melief et al., 2013; Naf-

tali-Shani et al., 2013; Ortiz et al., 2007), and the host microenvironment (Prockop, 2013). In view of these heterogeneities, there exists an urgent need to develop a platform for controlling the MSC secretome post-transplantation. To address this, we subjected hMSCs to a simulated inflammatory microenvironment by TNF- α stimulation (10 ng/ml) and characterized the secretome. It is important to consider that the levels of TNF- α vary in the host microenvironment, and thus MSC response cannot be easily predicted. The pro-inflammatory effect of TNF- α is generally mediated by NF- κ B. In view of this, we investigated whether NF- κ B inhibition in TNF- α -stimulated MSCs could inhibit the pro-inflammatory secretome sufficiently to suppress outcomes of inflammation. We demonstrate that the pro-inflammatory phenotype of hMSCs under an inflammatory stimulus can be transiently inhibited using intracellular release of a small-molecule inhibitor of NF- κ B (TPCA-1) via microparticles. This in turn is sufficient to suppress differentiation of CF to CMF.

In hMSCs, TNF- α mediates the upregulation of pro-inflammatory gene expression and protein secretion via NF- κ B (Carrero et al., 2012; Lee et al., 2010). Of 250 secreted factors profiled, we found that TNF-hMSCs secreted nine pro-inflammatory mediators at significantly higher concentrations compared with control-hMSCs. These mediators, barring RANTES (CCL5), are constitutively secreted, and TNF- α further augments their secretion levels. Secretion of RANTES, however, seems exclusively to be the result of TNF- α stimulation since it is not constitutively secreted.

The pro-inflammatory secretome from MSCs is reported to have deleterious effects in several disease models (Table S1). Pro-inflammatory mediators such as IL-6 and MCP-1 mediate differentiation of CF to CMF and collagen deposition, leading to adverse remodeling and heart failure. MCP-1 attracts circulating monocytes and induces differentiation to M1 macrophages, leading to collagen deposition and adverse fibrosis in aging hearts (Cieslik et al., 2014). Macrophages aggravate the inflammatory response by releasing elevated levels of pro-inflammatory cytokines including IL-6, MCP-1, IL-8 which in turn mediate differentiation of CF to CMF. Hence, therapeutic interventions are primarily aimed at reducing CMF differentiation thereby preventing irreversible scar formation and maximizing viable myocardium. The negative effects of pro-inflammatory mediators under disease settings such as cardiac fibrosis provided the insight for inhibiting their secreted levels from hMSCs to maximize the benefits of hMSC-based therapy.

Initiation and progression of cardiac fibrosis may occur due to secretion of TNF- α and IL-6 by infused MSCs (Naf-tali-Shani et al., 2013). Naive BM-MSCs can be pro-inflammatory and hence not sufficiently beneficial in improving ischemic myocardium and might require pretreatment to



THP-1 Human Monocytic Cells

Human CD14+ Primary Monocytes

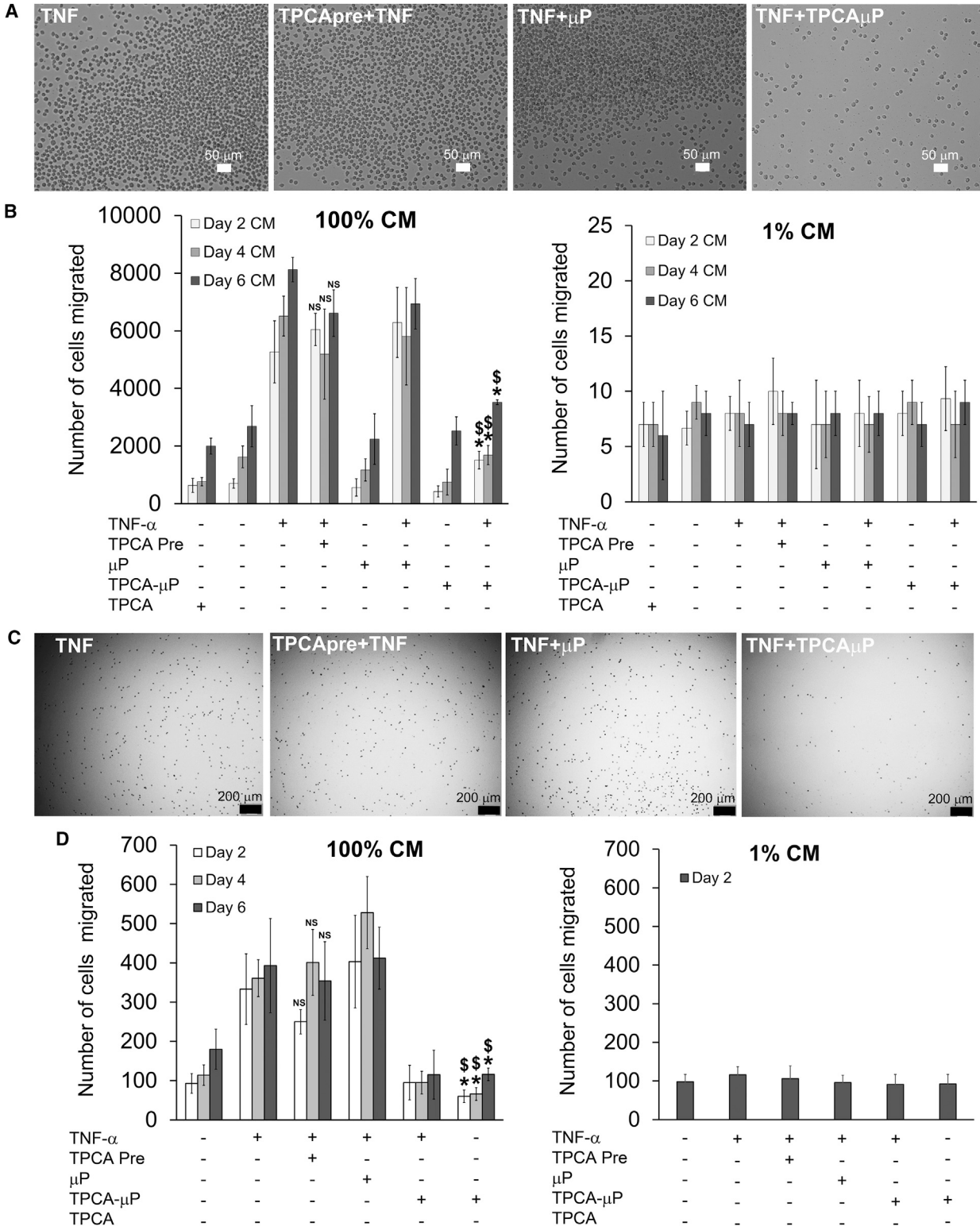


Figure 4. Dose-Dependent Transwell Migration of Human Monocytic THP-1 Cells and Human Primary Monocytes for 2 h in Response to Conditioned Medium from TPCA Preconditioned and Microparticle-Engineered hMSCs

(A) Representative bright-field images of migrated THP-1 cells in the bottom of the wells.

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maximize efficacy (Eun et al., 2011). For example, infused TNFR2 and TNFR1/R2 knockout mMSCs (into isolated rat hearts) after ischemia-reperfusion injury offered either no benefit or reduced cardioprotection whereas infused TNFR1 knockout mMSCs provided greater cardioprotection, measured via myocardial TNF- α levels and left ventricular diastolic performance compared with WT mMSCs. This suggests that by controlling TNFR signaling, MSC phenotype could be modulated between pro-inflammatory to anti-inflammatory (Kelly et al., 2010). TNF- α stimulation (10 ng/ml for 48 hr) also results in a significantly elevated concentration of pro-inflammatory mediators in the hMSC secretome (Lee et al., 2010) stimulating migration of human monocytes in vitro that might aggravate inflammation post-MI. Hence, we sought to non-genetically and transiently inhibit secretion levels of pro-inflammatory mediators from hMSCs under TNF- α stimulation.

TPCA-1 reversibly binds to IKK β and prevents it from phosphorylating I κ B α for degradation and thereby blocks nuclear translocation of NF- κ B (Kondo et al., 2008). Our results demonstrate that TPCA-1 may inhibit the stimulatory action of TNF- α on NF- κ B. TPCA-1 partially inhibits secretion of constitutively secreted pro-inflammatory factors such as IL-6 and MCP-1, which suggests that constitutive secretion is not entirely dependent on NF- κ B activation. The reduction of RANTES, GRO, and GCP-2 to basal levels may indicate that NF- κ B activation mediates the secretion of these cytokines upon TNF- α treatment as TPCA-1 abrogates NF- κ B activation. Our cytokine array results confirm that TPCA-1 abrogates the effect of TNF- α on production of pro-inflammatory cytokines by hMSCs. In addition, we demonstrated that TPCA-1 treatment did not inhibit secretion of beneficial paracrine factors such as growth factors, cytokines, and anti-inflammatory mediator TSG-6, which when secreted from lung embolized hMSCs attenuate MI in mice (Lee et al., 2009). Hence, angiogenic and TSG-6-mediated anti-inflammatory properties of hMSCs may not be compromised upon TPCA-1 treatment. In addition, continuous intracellular TPCA exposure to hMSCs might yield sustained binding to IKK β , resulting in the inhibition of NF- κ B and overcome reversible inhibition of IKK β . TPCA-1 has non-specific effects of IKK β inhibition on many cell types with potential undesirable side effects. In addition, MSCs demonstrate a “hit and run effect” of immune modulation (Levy et al., 2013) and are cleared from the body within a week post-

transplantation. Hence it is desirable to intracellularly deliver TPCA-1 into hMSCs at a controlled rate for transient modulation of the pro-inflammatory secretome.

In this study, we developed an engineering platform to intracellularly deliver TPCA-1 to hMSCs. μ P s of 1–2 μ m were internalized following anionic surface modification. By choosing PLGA polymer with a lactic acid to glycolic acid ratio of 50:50 and low molecular weight of 10 kDa, we tailored the release kinetics of TPCA-1 to achieve a concentration to inhibit IKK2. Upon preconditioning of hMSCs with soluble TPCA prior to stimulation with TNF- α , the hMSC secretome was similar to TNF- α stimulation alone (TNF-hMSC) suggesting that IKK β inhibition may require continued presence of TPCA to prevent NF- κ B activation. The sustained intracellular release of TPCA-1 enabled prolonged inhibition of IKK β and competitively inhibited TNF- α from activating NF- κ B mediated pro-inflammatory cytokine secretion. NF- κ B signaling in cells is oscillatory in nature, and gene expression seems to depend on its dynamics (Nelson et al., 2004). Hence, under continuous TNF- α stimulus, NF- κ B activation might be sustained. However, the effect of intracellular TPCA inhibition in microparticle-engineered hMSCs was maintained after multiple rounds of TNF- α stimulation suggesting that microparticle-engineered hMSCs are able to control the pro-inflammatory phenotype under sustained inflammatory stimulus.

To demonstrate the functional relevance of microparticle-mediated hMSC secretome regulation, we focused on cardiac fibrosis. IL-6 (Ma et al., 2012), MCP-1, and other pro-inflammatory mediators augment monocyte migration (Lee et al., 2010) and induce differentiation of CF to CMF, which are the primary scar-forming cells post-MI. Expectedly, we found that while the elevated levels of pro-inflammatory mediators in CM from preconditioned hMSCs are ineffective in inhibiting monocyte migration, CM from TNF + TPCA μ P-hMSCs effectively inhibited transwell migration of THP-1 cells by at least 2- to 3-fold, corresponding to reduced levels of pro-inflammatory mediators as seen by our secretome analysis.

Control-hMSC CM also induced significant differentiation of CF to CMF demonstrating the presence of pro-inflammatory mediators secreted by hMSCs as well as those likely to be present in the culture serum. CM from control-hMSCs and μ P-hMSCs have lower levels of pro-inflammatory factors (compared with TNF- α -stimulated

(B) Quantitative analysis of the number of THP-1 cells migrated into the bottom well in response to 100% CM or 1% CM from TPCA-1 preconditioned and microparticle-engineered hMSCs. Data represent the average of triplicates from three independent experiments.

(C) Representative bright-field images of migrated primary macrophages in the bottom of the wells.

(D) Quantitative analysis of the number of primary macrophages migrated into the bottom well in response to 100% CM or 1% CM from TPCA-1 preconditioned and microparticle-engineered hMSCs.

Data represent the average of triplicates from three independent experiments. NS represents non-significance versus TNF-hMSC CM, whereas * represents $p < 0.01$ versus T-hMSC CM and \$ represents $p < 0.01$ versus μ P + TNF-hMSC CM. Error bars represent \pm SD.

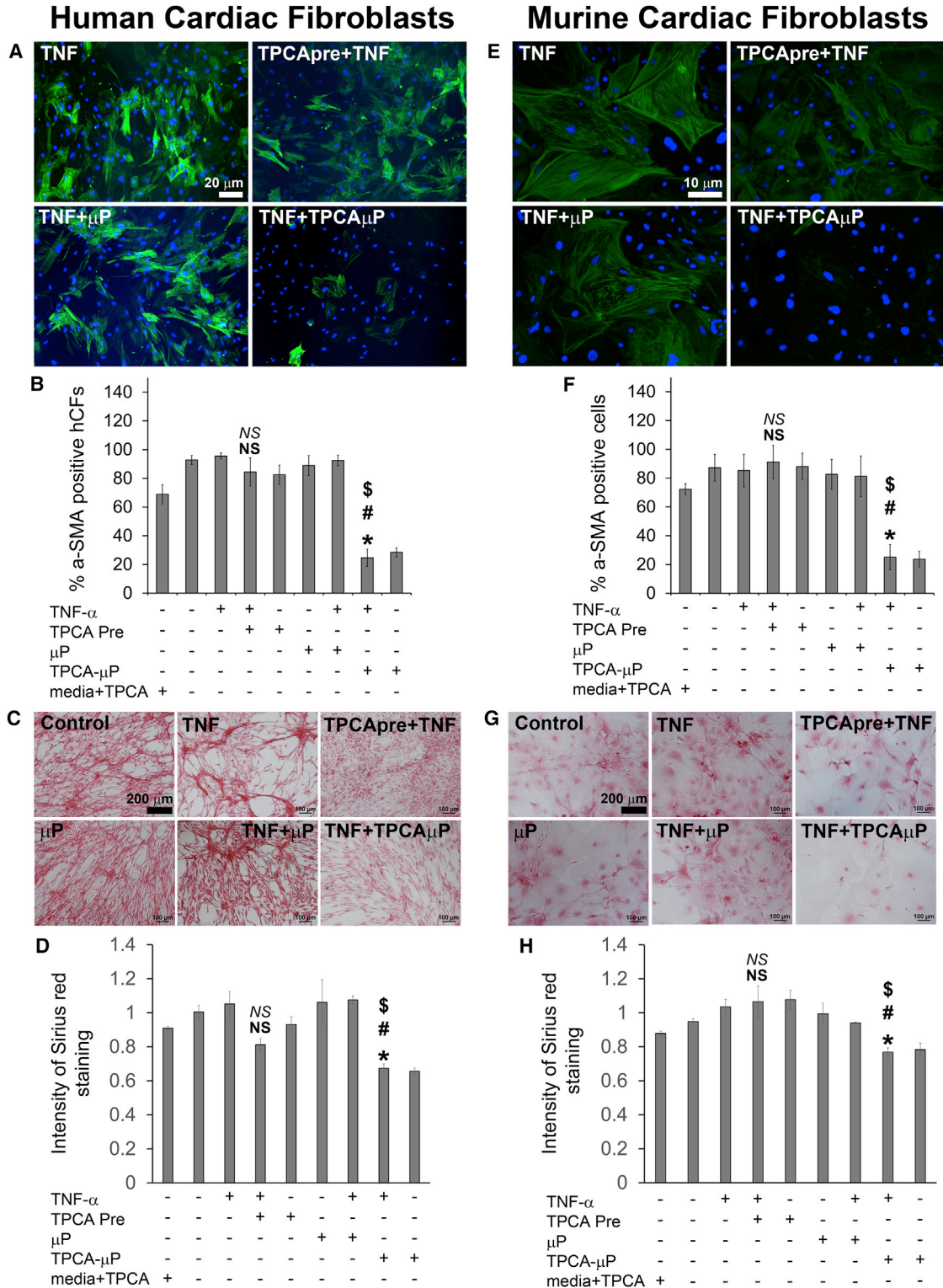


Figure 5. Analysis of α -SMA Expression and Collagen Deposition of Human and Murine CF in Response to Conditioned Media of TPCA Preconditioned and Microparticle-Engineered hMSCs

(A) Representative fluorescence images (4 \times) of hCF treated with day 2 CM from each hMSC group (green, α -SMA; blue, DAPI).

(B) Quantitative analysis of the number of α -SMA + hCF treated with day 2 CM from each hMSC group.

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hMSCs) yet the number of α -SMA⁺ CMF remained unchanged when CF were treated with these CM, suggesting that the secretome may lack anti-inflammatory modulators and growth factors. In addition, treatment with TPCA-1 alone did not cause a reduction in the number of α -SMA⁺ CMF, suggesting that an hMSC-mediated paracrine function is essential. While TPCA-1 pretreatment of hMSCs before TNF- α stimulation showed a significant reduction in CMF numbers, this was greatly enhanced when TPCA-1 was available intracellularly via the microparticles. CM from TNF + TPCA μ P-hMSC likely prevented differentiation of CF to CMF due to the continued intracellular inhibition of IKK-mediated NF- κ B activation, thus preventing the release of an hMSC pro-inflammatory secretome. Surprisingly, the CMF number was reduced by over 2-fold, suggesting that TPCA-1 may activate hMSC pathways that revert the CMF phenotype. We cannot rule out the possibility that other contributors in the hMSC secretome that were not profiled might also be contributing, and their action is facilitated by intracellular TPCA-1. For instance, in an in vitro 3D model of cardiac fibrosis under hypoxic conditions, reversal of CMF to the CF phenotype was shown to be due to reduced MSC TGF- β levels (Galie and Stegemann, 2014). Our assay revealed a similar trend in terms of collagen production from CF. CF treated with CM from control-hMSCs, or TPCApre + TNF-hMSCs or μ P-hMSCs secreted elevated levels of collagen into the media suggesting that only inhibited levels of pro-inflammatory mediators could not be implicated in the reduction in collagen secretion. The reduced number of α -SMA⁺ CMF in CM from TNF + TPCA μ P-hMSCs possibly contributed to the lower secretion level of collagen in the media (Figure 5B). Upon de-differentiation or lowered α -SMA expression, it is possible that CMFs lose collagen secretion ability. In regions of MI, CF switch to the myofibroblast phenotype due to stress from the infarct scar (Tomasek et al., 2002). High expression of α -SMA typical in such CMF (Teunissen et al., 2007) has been implicated for remodeling due to their high contractility (Santiago et al., 2010). In addition, the collagen secretion capacity of CMF is very high (Petrov et al., 2002). Overall, attenuation in the number of collagen-secreting α -SMA⁺ CMF could be beneficial in

preventing pathological remodeling or irreversible scar formation and allowing cardiac regeneration.

Here we have demonstrated that the pro-inflammatory hMSC secretome could be inhibited using a microparticle engineering approach delivering an intracellular NF- κ B inhibitor, TPCA-1. It is however important to note that the MSC secretome composition may change depending on the level of TNF- α encountered in vivo following transplantation. Nevertheless, a similar approach could be beneficial in inflammatory disease settings such as chronic inflammation and macrophage-mediated atherosclerosis. The approach of microparticle engineering of an exogenous cell population by modulating a central regulatory pathway, may find application in other cell types and pathways and could provide an attractive strategy for harnessing any cell secretome for therapy. This approach could also be potentially employed to modulate the composition of extracellular vesicles (exosomes) for therapy.

EXPERIMENTAL PROCEDURES

Mesenchymal Stromal Cell Culture and Characterization

Human MSCs and human and murine CF culture and characterization are described in [Supplemental Experimental Procedures](#).

Stimulation of hMSCs with TNF- α and TPCA-1 Treatment

Primary hMSCs derived from bone marrow of healthy consenting donors were cultured in standard media and used at passage number 3–6 (see [Supplemental Experimental Procedures](#)). 30,000 cells per well in a 24-well plate were cultured in serum-free MesenCult XF media (STEMCELL Technologies) for 24 hr and then treated for 48 hr with either (1) 0.1% (v/v) DMSO (Sigma) (control-hMSC), or (2) 10 ng/ml TNF- α (Sigma-Aldrich) (TNF-hMSC), or (3) 10 μ M TPCA-1 2-[(aminocarbonyl)amino]-5-(4-fluorophenyl)-3-thiophenecarboxamide (Calbiochem), or (4) 10 μ M TPCA-1 and stimulating with 10 ng/ml TNF- α (TNF + TPCA-hMSC), followed by conditioned media (CM) collection. Metabolic activity of cells was measured using the MTS assay (see [Supplemental Information](#)).

Secretome Profiling of Stimulated hMSCs

Quantitative measurement of 250 human cytokines in the stimulated hMSC CM was performed using Quantibody Human

(C) Representative bright-field images of hCF treated with day 2 CM from each hMSC group.

(D) Quantitative analysis of relative Sirius red staining intensity from hCF in response to CM from each hMSC group.

(E) Representative fluorescence images (10 \times) of mCF treated with day 2 CM from each hMSC group (green, α -SMA; blue, DAPI).

(F) Quantitative analysis of number of α -SMA⁺ mCF treated with CM from each hMSC group.

(G) Representative bright-field images of mCF treated with day 2 CM from each hMSC group.

(H) Quantitative analysis of relative Sirius red staining intensity from mCF in response to day 2 CM from each hMSC group.

NS represents not significant versus hMSC media + TPCA; NS represents not significant versus TNF-hMSC CM; * indicates $p < 0.01$ versus hMSC media + TPCA; \$ indicates $p < 0.01$ versus TPCApre + TNF-hMSC CM; # indicates $p < 0.01$ versus TNF + μ P-hMSC CM. All data represent the average of triplicates from three independent experiments from two different donor hMSCs. Error bars represent \pm SD.



Cytokine Array 5000 (Ray Biotech) according to the manufacturer's instructions. Fluorescence signals at 532 nm from the arrays were obtained by scanning the slides using an Axon GenePix 4300A microarray laser scanner (Molecular Devices). Spot intensity was quantified into concentration values using standard curves based on linear regression and analyzed (fold change in concentration) using GenePix Pro 7 software. See [Supplemental Information](#) for details.

PLGA Microparticle Fabrication, Characterization, and Engineering of hMSCs

TPCA-1, or Rhodamine 6G dye (Sigma) or DiI dye (Cell Signaling) were encapsulated in PLGA microparticles using the single-emulsion technique. To enhance microparticle uptake and loading in hMSCs, the surface of PLGA microparticles was modified as described before ([Ankrum et al., 2014b](#)). See [Supplemental Information](#) for details.

Analysis of Microparticle-hMSC Interaction

DiO-loaded-microparticle internalized hMSCs (D μ P-hMSCs) were trypsinized and analyzed by flow cytometry to characterize the association of microparticles with hMSCs. The mean fluorescence of the gated cell population was quantified. hMSCs with no particles and blank particles served as controls. D μ P internalization was characterized by confocal microscopy imaging (Zeiss LSM510) of fixed cells followed by image analysis. Microparticles within the plane of the nucleus and inside the boundaries of the cell membrane were interpreted to be internalized. Cytotoxicity of TPCA μ Ps on hMSCs was measured using the MTS assay as described earlier.

Release Kinetics of TPCA-1 from Microparticles

In vitro release kinetics of TPCA-1 from TPCA μ Ps was profiled by suspending 6 mg of TPCA μ Ps in 1 ml of PBS and loading into a 1.5 × 6 cm polytetrafluoroethylene dialysis membrane (Thermo Fisher Scientific) with 8–10 kDa molecular weight cut off. The membrane was then introduced into 20 ml of PBS and incubated at 37°C with shaking. PBS was sampled at specific time points and drug was extracted using dichloromethane (DCM). Following evaporation of DCM and dissolution of the drug in DMSO, the concentration of TPCA-1 was measured by determining absorbance at 320 nm using a UV-visible spectrophotometer.

Analysis of the Composition and Kinetics of Preconditioned and Microparticle-Engineered hMSC Secretome

The experimental procedure is described in [Figure 3A](#). The effect of TPCA preconditioning or internalized TPCA μ Ps on TNF- α -treated hMSC secretome was investigated by performing an ELISA of CM to quantify MCP-1, IL-6, and RANTES concentrations. See [Supplemental Information](#) for details.

Statistical Analysis

Single-factor ANOVA was used for multiple pairwise comparisons with two-factor t test with Bonferroni correction post hoc analysis. $p < 0.01$ was considered significant.

See [Supplemental Information](#) for additional methods.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, five figures, and one table and can be found with this article online at <http://dx.doi.org/10.1016/j.stemcr.2016.05.003>.

AUTHOR CONTRIBUTIONS

S.H.R. and O.L. designed experiments and contributed to discussions; S.H.R., Z.T., and K.M., performed experiments. J.M.K. and M.S.I. conceptualized and supervised the experiments. S.H.R., J.M.K., and M.S.I., wrote the paper.

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