**Supplemental Material**

**Expanded Methods**

*Valvular Interstitial cell (VIC) isolation and culture*

Primary porcine valvular interstitial cells (VICs) isolated from aortic heart valves as described previously.(1) Briefly, hearts were acquired from pig (10 months), which were sacrificed at an USDA approved abattoir (THOMA Meat Market, Saxonburg, PA). To remove endothelial cells valve surfaces were scraped three hours after dissection. VIC isolation was performed using collagenase A (Sigma, St. Louis, MO) digestion. Cells were cultured in normal growth medium containing Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum and 1% penicillin/streptomycin (control medium) (Invitrogen, Grand Island, NY) at 37 °C, 5% CO2. Cells between passage 3 and 6 were used for all experiments.

*Synthesis of materials*

HAMA was synthesized as reported previously.(2) Briefly, after making a solution of 1wt% hyaluronic acid (Lifecore Biomedical, Chaska, MN) in deionized water, methacylic anhydride (Sigma-Aldrich, St. Louis, MO) was added. By adding 5M NaOH (Sigma-Aldrich, St. Louis, MO), the pH was adjusted to 8, during which the solution was kept on ice for 24 hours. After methacrylation the HAMA solution was dialyzed against deionized water for 72 hours, which was followed by lyophilization. This resulted in solid white foam like material, which was stored at -80 °C before using it for experiments.

The synthesis of GelMA has also been described before.(3) In brief, powdered type A cell culture tested gelatin from porcine skin (Sigma-Aldrich, St. Louis, MO) was dissolved in phosphate buffered saline (PBS), and then heated to 60 °C under continuous stirring for 20 minutes to obtain a 10 wt% gelatin solution. Metacrylated gelatin (GelMA) solution was formed by drop wise adding of 8% (v/v) methacrylic anhydride under constant stirring for 3 hours at 50 °C. The GelMA solution was then diluted and dialyzed against deionized water at 40 °C for one week. Subsequently, the solution was lyophilized for 96 hours which yielded a white porous foam-like material, which was stored at −80 °C before experimental use.

*Hydrogel fabrication*

Hybrid hydrogels were fabricated from HAMA and GelMA using photocrosslinking as previously reported.(1, 4) Briefly, 1mg photo-initiator (PI) (Igracure 2595) was dissolved in 1 mL of phosphate buffered saline (PBS; Gibco) at 80 °C. To achieve a concentration of 1wt% HAMA and 5wt% GelMA, we added 1 mL PI solution to 10 mg HA and 50 mg GelMA and put this into an 80 °C oven for 20 minutes. The solutions were vortexed regularly. The polymer solution was then allowed to cool off to 37 °C. VICs at 80% confluency were trypsinized and centrifugated at 1500 rpm for 5 minutes. The supernatant was aspirated and the remaining cell pellet resuspended in the prepolymer solution. We used a cell density of 10 million cells/mL. 50 µL of the cell-laden prepolymer solution was drop-wise added to a petri dish between two spacers with a height of 450 µm and covered with an autoclaved sterile glass slide. The cell-laden solution was then subjected to light (wavelength 360 nm) for 30 seconds with an intensity

of 450 mW at a 10 cm height yielding a light intensity of 2.5 mW/cm$^2$. The exposure to light facilitated photoinitiator dependent crosslinking of the polymers in the solution yielding a disc shaped hydrogel with a height of 450 μm. The VIC-laden hydrogel was removed from the glass slide and placed into a well plate containing either control medium (CM) or osteogenic medium (OM) (control growth medium supplemented with 10 mM β-glycerophosphate, 10 ng/mL ascorbic acid, and $10^{-8}$ M dexamethasone) or OM with tumor necrosis factor alpha (TNF-α). The VIC-laden hydrogels were cultured for up to 21 days.

*Silencing of alpha Smooth Muscle Actin in VIC-laden hydrogels*

To inhibit the expression of α-smooth muscle actin (α-SMA), ON-TARGET plus SMART-pool (L-003605-00-00100) and a negative control (scramble (SCR)) from Dharmacon RNAi Technologies/ThermoScientific (Chicago, IL, USA) was used. Transfection of 50 nmol/l siRNAs was performed using DharmaFECT 1 transfection reagent twice per week over the entire culture period. Samples were analyzed at day 14 and day 21 of culture.

*Histological analysis and immunofluorescence staining of VIC-laden hydrogel constructs*

Human calcified aortic valves were obtained from CAVD patients that underwent surgical valve replacement surgeries according to Brigham and Women's Hospital IRB protocol (#201-P-002567/2; BWH). Tissue was frozen in optimal cutting temperature (OCT) compound (Sakura Finetech, Torrance, CA), of which 7 μm sections were cut. The VIC-laden hydrogels were washed with PBS for 5 minutes and then fixed in 4%

paraformaldehyde for 20 minutes, followed by PBS wash. The hydrogels were placed into a 30% (w/v) sucrose solution overnight at 4 °C, after which they were frozen in OCT compound and 10μm sections were cut. To detect calcium deposition, sections were stained with 0.02 mg/mL Alizarin Red S (ARS) solution (Sigma, St. Louis, MO). Von Kossa staining (American MasterTech) was used to visualize phosphate deposition in the VIC-laden hydrogel cross sections. Images were obtained using Nikon Elipse microscope (Nikon Instruments). Calcific nodules/ positively stained cells were quantified by manually counting noduli per high power field (HPF) using five fields per hydrogel, and three hydrogels per time point for each condition.

Immunofluorescence staining for α-SMA and runt-related transcription factor 2 (Runx2) was performed. Hydrogel sections were permeabilized using 0.1% Triton-X. After blocking in 4% horse serum, sections were incubated with monoclonal mouse anti-human α-SMA primary antibody (Clone 1A4, Dako, Dako Denmark A/S, Glostrup, Denmark) or a monoclonal mouse anti-Runx2 primary antibody (Abcam, Cambridge, USA) for 90 minutes at room temperature (RT), followed by biotin labeled secondary antibody (Vector Labs, Burlingame, CA, USA) for 45 minutes at RT and streptavidin labeled AlexaFluor 488 (Invitrogen, Grand Island, NY) for α-SMA and AlexaFluor 594 (Invitrogen, Grand Island, NY) for Runx2. Sections were washed three times in PBS for 5 minutes and nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI) containing mounting medium (Vector Labs, Burlingame, CA, USA). Images were taken with an Eclipse 80i microscope (Nikon, Melville, NY, USA) and processed with Elements 3.20 software (Nikon, Melville, NY, USA). Positive staining was quantified by

manually counting positively stained cells of the total cell number in five HPF per hydrogel. Three hydrogels were quantified per condition for each time point.

*Quantification of alkaline phosphatase and calcium in engineered hydrogel constructs*

Alkaline phosphatase (ALP) activity was measured using a colorimetric kit (Biovision Lifesciences, Milpitas, CA, USA). Samples were washed in PBS, and stored in ultrapure water at -80 °C before use. Three hydrogels per time point per experiment were pooled together. Upon analysis, samples were thawed and homogenized using a tissue lyzer (Qiagen, Venlo, The Netherlands). The homogenized hydrogels were centrifuged at 13.000 rpm for 3 minutes. The supernatant containing ALP was transferred to new tubes. To measure ALP activity we followed the manufacturer's protocol. Briefly, 16 µL of supernatant was added to 64 µL of ALP assay buffer and 50 uL of PnPP solution was added and incubated for 1 hour at room temperature. The absorbance was read at a wavelength of 405 nm. Values were normalized to the standard curve. Calcium content was quantified using a colorimetric kit (Biovision Lifesciences, Milpitas, CA, USA), using the manufacturer's protocol. Briefly, 50 µL of sample was added to 60 µL of calcium assay buffer, followed by 90 µL chromogenic reagent is added and incubation for 10 minutes at room temperature in the dark. Absorbance was read at a wavelength of 575nm with a plate reader. Values were normalized to the standard curve. Calcium content and ALP activity were normalized to DNA content. Double stranded DNA (dsDNA) was measured with a Quanti-it PicoGreen dsDNA Quantification Kit (Invitrogen, Grand Island, NY, USA).

*Real Time Polymerase Chain Reaction for expression of cell markers*

VICs were isolated from cell-laden hydrogels using a mechanical disruption of the hydrogels (TissueLyzer, Qiagen, Venlo, The Netherlands). Total RNA was isolated from using GE Healthcare RNAspin mini RNA Isolation kit. The amount of RNA in each sample was measured using NanoDrop 2000c (ThermoScientific, Waltham, MA, USA). Total RNA was reverse transcribed with oligo-(dT)12-18 primers(Invitrogen/Life Technologies,Grand Island, NY, USA) and Superscript II reverse transcriptase (Invitrogen/Life Technologies,Grand Island, NY, USA) to obtain a target cDNA concentration of 0.335ug/mL followed by RT- PCR using SYBR Green (BioRad, Hercules, CA, USA), and annealing temperatures of 95 and 60 degrees Celsius for 35 cycles. Primer sequences were designed with Primer3 software and were as follows: α-SMA: F:5'-AGTGCGACATTGACATCAGG -'3 and R:5'-CTGGAAGGTGGACAGAGAGG -'3, Runx2 F:5'- ACCCAGAAGACTGTGGATGG -'3 and R:5'- ACCTGGTCCTCAGTGTAGCC -'3 The housekeeping gene used was Glyceraldehyde-3-phosphate dehydrogensase (GAPDH): F:5'-CCCAGAAGACTGTGGATGG -'3 , R:5'- ACCTGGTCCTCAGTGTAGCC -'3. Expression was quantified using comparative Ct (Cycle threshold method) $2^{-\Delta\Delta CT}$ method) with the following equations: (1) ΔCT =CT of target gene –CT of housekeeping gene, (2) ΔΔCT=ΔCT day x – ΔCT day 1; (3) Fold increase between groups = $2^{-\Delta\Delta Ct}$

*Cell viability and apoptosis*

Cell viability was determined by fluorescent labeling with 4μM Calcein AM and 2μM Ethidium Homodimer-1 (LIVE/DEAD Viability kit for mammalian cells, Invitrogen, Grand Island, NY, USA). Cell-laden hydrogels were first washed with PBS for 5 minutes and then incubated with fluorescent dye for 20 minutes at room temperature. The cell-laden hydrogels were then washed with PBS again and imaged using an A1/C1 confocal microscope (Nikon Insruments, Inc. Melville, NY) Three hydrogels were analyzed per time point per condition. Of each hydrogel 3 z-stacks (10 μm per slice) were made of which a compressed image was formed. Amount of viable cells were stained green and dead cells red, and were manually counted using Image J Software. Data is depicted as percentage of live cells of the total cell number. Apoptosis was determined by TUNEL (terminal deoxynucleotidyl transferase mediated dUTP nick end labeling) staining (Millipore, Remecula, CA, USA), according to manufacturer's protocol and quantified using fluorescence microscopy. Data are depicted as percentage of positively stained cells.

*Statistical Analysis*

Results are presented as mean +/- standard deviation unless indicated otherwise. Unpaired student's t-test was used for comparisons between two groups. One-way ANOVA was used to evaluate statistical significant differences in multiple groups. $P < 0.05$ was considered significant.

**Supplemental Figure Legends**

**Figure Supplemental I. Calcification potential of 3D *in vitro* valve model.** 40 x magnification of Von Kossa staining of VIC laden hydrogels cultured in osteogenic media (OM) or osteogenic media with TNF-α (OM + TNF-α) at day 21. Arrows indicate brown/black positive staining. Bar: 50μm

**Figure Supplemental II. ALP Activity in 3D constructs.** ALP activity, a marker for early calcification was measured every 72 hours in VIC laden hydrogels cultured in osteogenic media (OM) and osteogenic media with TNF-α. Data is depicted as mean ± SD (n=3)

**Figure Supplemental III. Cell viability after 21 days of culture.** VIC-laden hydrogels were cultured in control media (CM) and osteogenic media (OM) for up to 21 days. **A,** Representative confocal images of the middle of the VIC-laden hydrogel stained with Live/Dead assay. Live cells are green, dead cells red. Bar = 100 μm **B,** Quantification of cell viability, normalized for cell number. Data is depicted as mean percentage live cells ± SD (n=3)**.**

**Figure Supplemental IV. Evaluation of apoptosis in VIC-laden hydrogels**. **A,** TUNEL staining of VIC laden hydrogels at day 7, 14 and 21 after culture in osteogenic media (OM) and osteogenic media with TNF-α (OM + TNF-α). Arrow indicates positive staining for apoptosis. Bar=50 μm. **B,** Quantification of percent negative apoptotic cells using Image J. (n=3) Data is depicted as mean ± SD.

**Figure Supplemental V. Quantification of α-SMA positive cells .** VIC-laden hydrogels were cultured osteogenic media (OM) and osteogenic media with TNF-α (OM +TNF-α) for up to 21 days. **A,** Quantification of positively stained cells for α-SMA at each time point. Data

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**Figure Supplemental VII. Early marker for osteogenesis is reduced when by silencing of α-SMA.** Alkaline phosphatase (ALP) activity normalized for DNA (ug/mL) content at day 14. Cell laden hydrogels were cultured for 14 days and either treated with SiRNA (SI-α-SMA) against α-SMA or scramble control (SCR) twice a week. (n=3). Data is depicted as mean ± SD. *$p < 0.05$.

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**Figure Supplemental IX. VIC phenotypes from four human donors of CAVD.** Representative immunofluorescent images and quantification for each of the four donors showing Runx2 (green) and α-SMA (red) with DAPI (blue) stained nuclei of calcified aortic heart valve.

**References**

1. Hjortnaes JC-U, G.; Hutcheson, J.D.;J ung, S.M, Schoen, F.J. ; Kluin, J.; Aikawa, E; Khademhosseini, A. A Hydrogel-based Platform to controllably direct Valvular Interstitial Cells fate. Submitted. 2013.

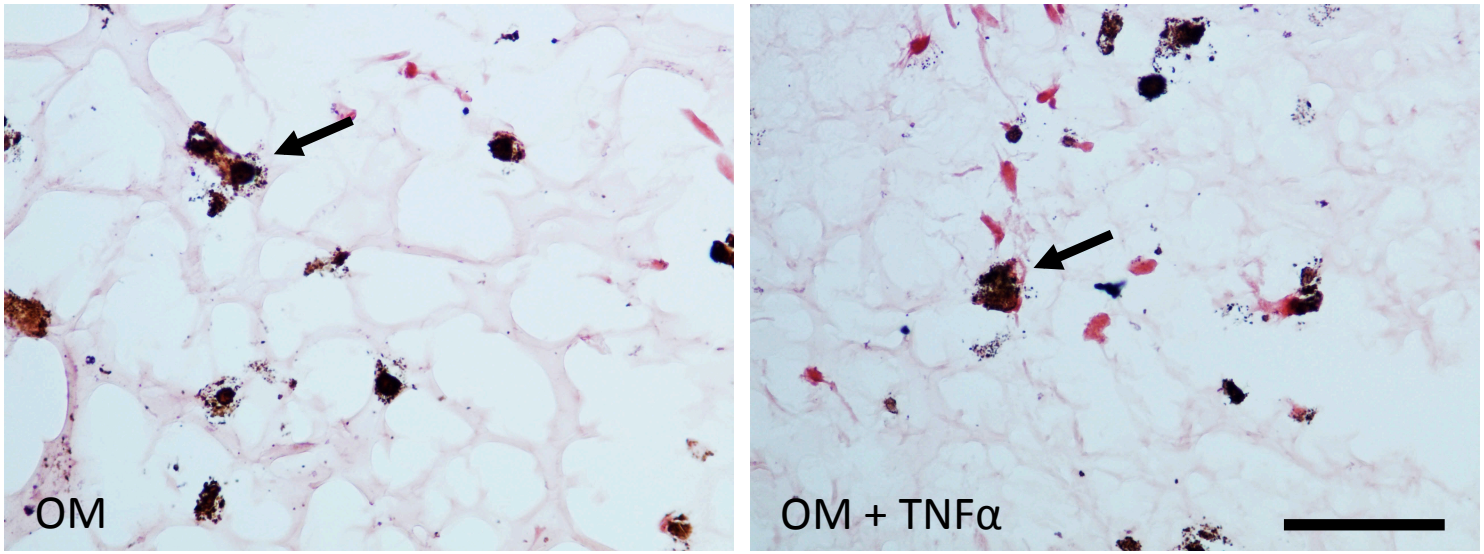
2. Camci-Unal G, Nichol JW, Bae H, Tekin H, Bischoff J, Khademhosseini A. Hydrogel surfaces to promote attachment and spreading of endothelial progenitor cells. Journal of tissue engineering and regenerative medicine. 2012. Epub 2012/01/10.

3. Ramon-Azcon J, Ahadian S, Obregon R, Camci-Unal G, Ostrovidov S, Hosseini V, et al. Gelatin methacrylate as a promising hydrogel for 3D microscale organization and proliferation of dielectrophoretically patterned cells. Lab on a chip. 2012;12(16):2959-69. Epub 2012/07/10.

4. Camci-Unal G, Cuttica D, Annabi N, Demarchi D, Khademhosseini A. Synthesis and characterization of hybrid hyaluronic acid-gelatin hydrogels. Biomacromolecules. 2013;14(4):1085-92. Epub 2013/02/20.

# Supplemental Figures

Figure I.



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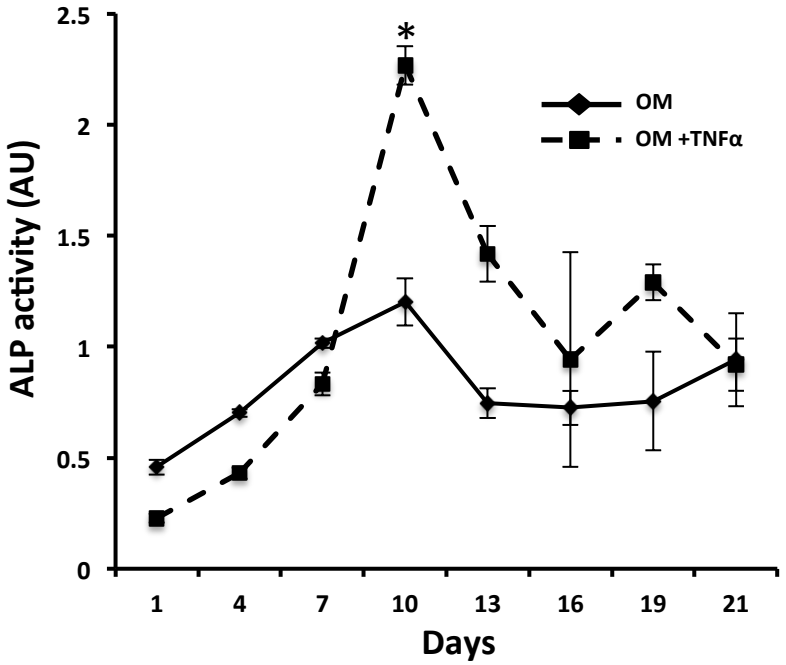

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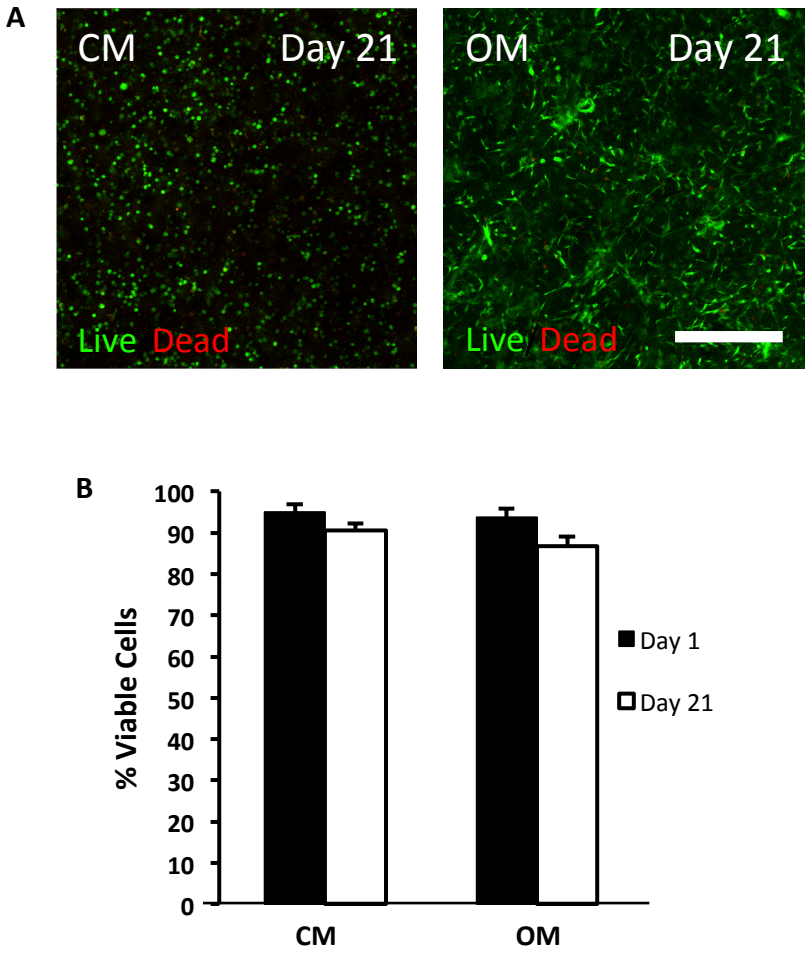

A
CM
Day 21
Live Dead
OM
Day 21
Live Dead
B
% Viable Cells
100
90
80
70
60
50
40
30
20
10
0
CM
OM
Day 1
Day 21

Figure IV.

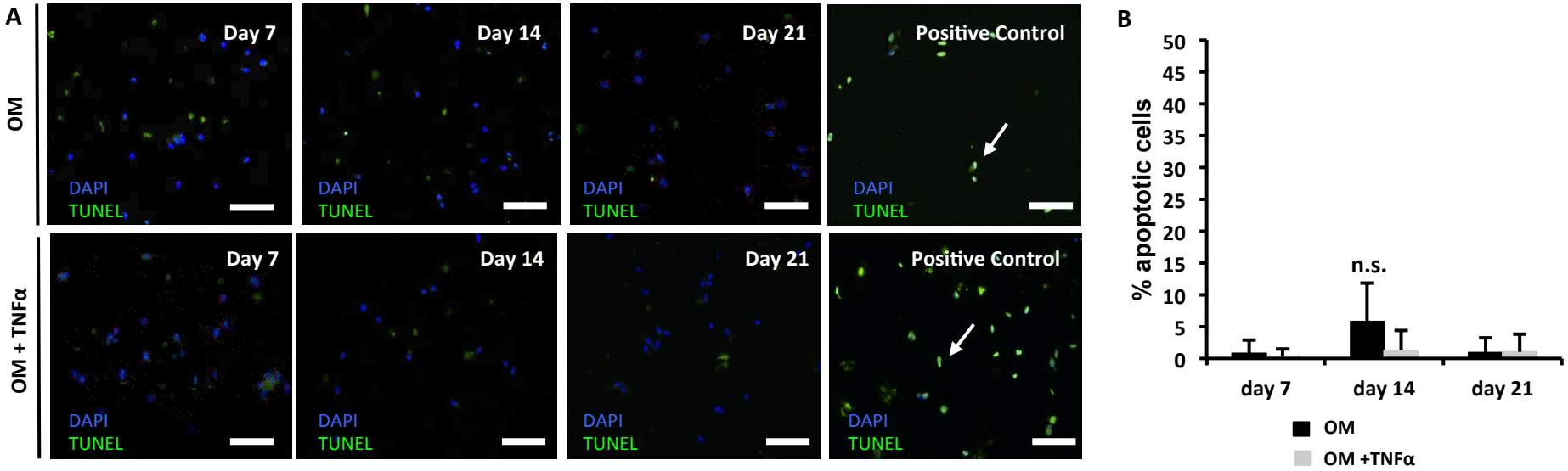

A
OM
OM + TNFα
Day 7
Day 14
Day 21
Positive Control
DAPI
TUNEL
B
% apoptotic cells
50
45
40
35
30
25
20
15
10
5
0
n.s.
day 7
day 14
day 21
OM
OM +TNFα

Figure V

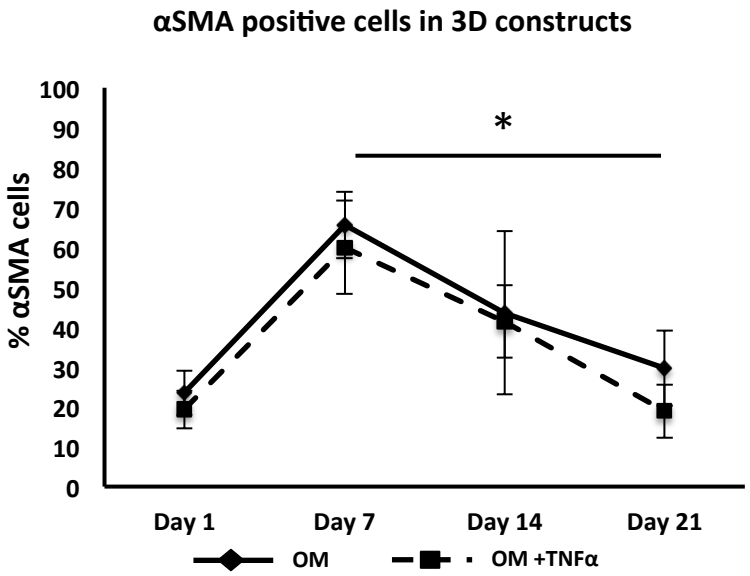

αSMA positive cells in 3D constructs
% αSMA cells
100
90
80
70
60
50
40
30
20
10
0
*
Day 1
Day 7
Day 14
Day 21
OM
OM +TNFα

Figure VI

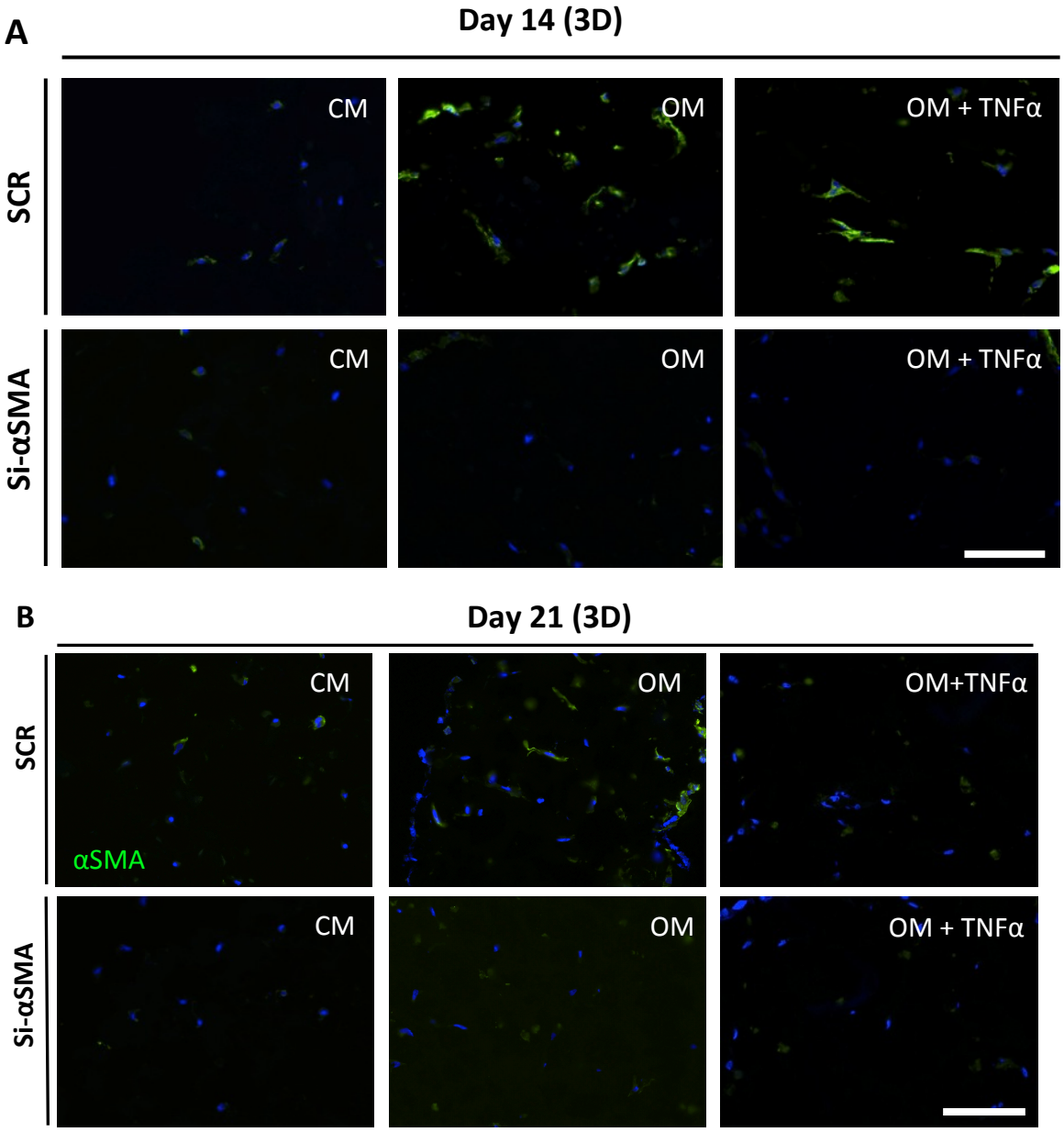

A
Day 14 (3D)
SCR
Si-αSMA
CM
OM
OM + TNFα
CM
OM
OM + TNFα
B
Day 21 (3D)
SCR
Si-αSMA
αSMA
CM
OM
OM+TNFα
CM
OM
OM + TNFα

Figure VII

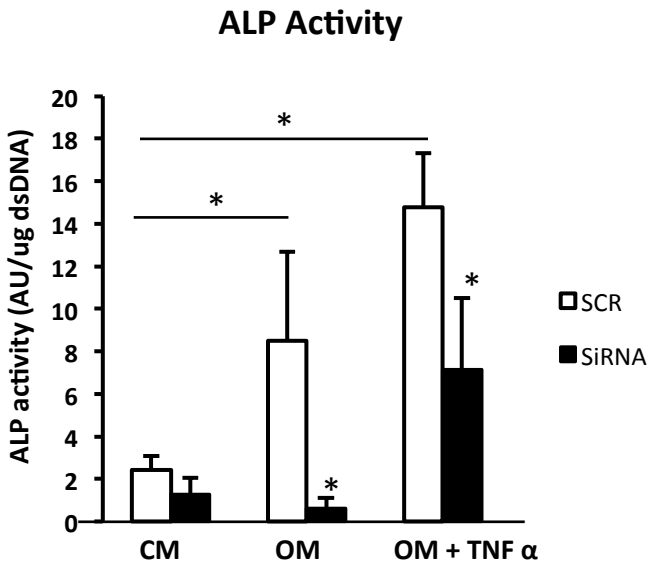
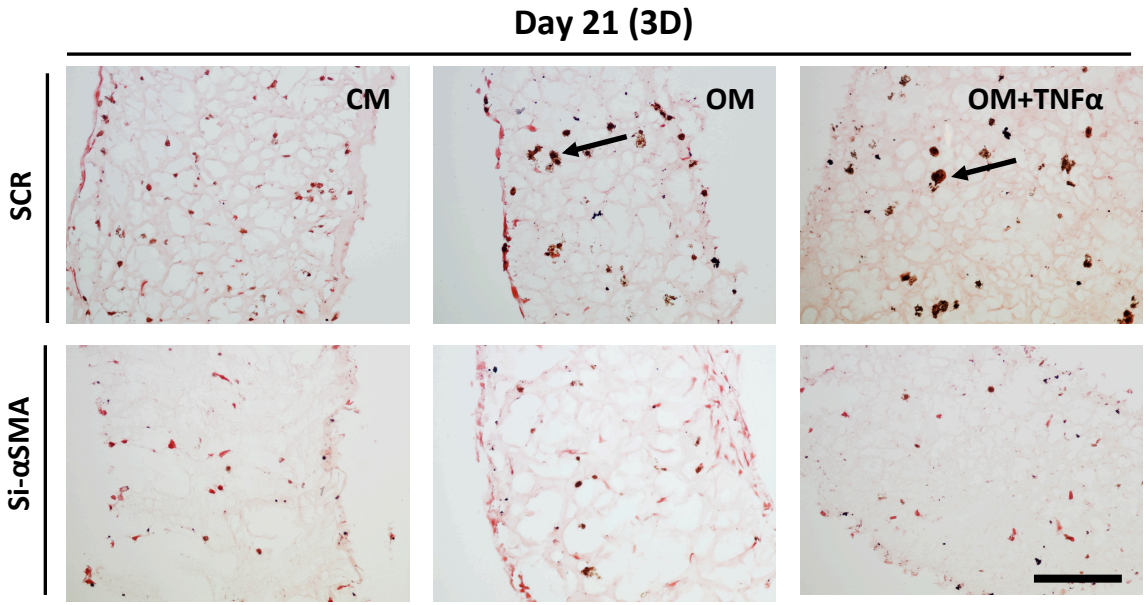
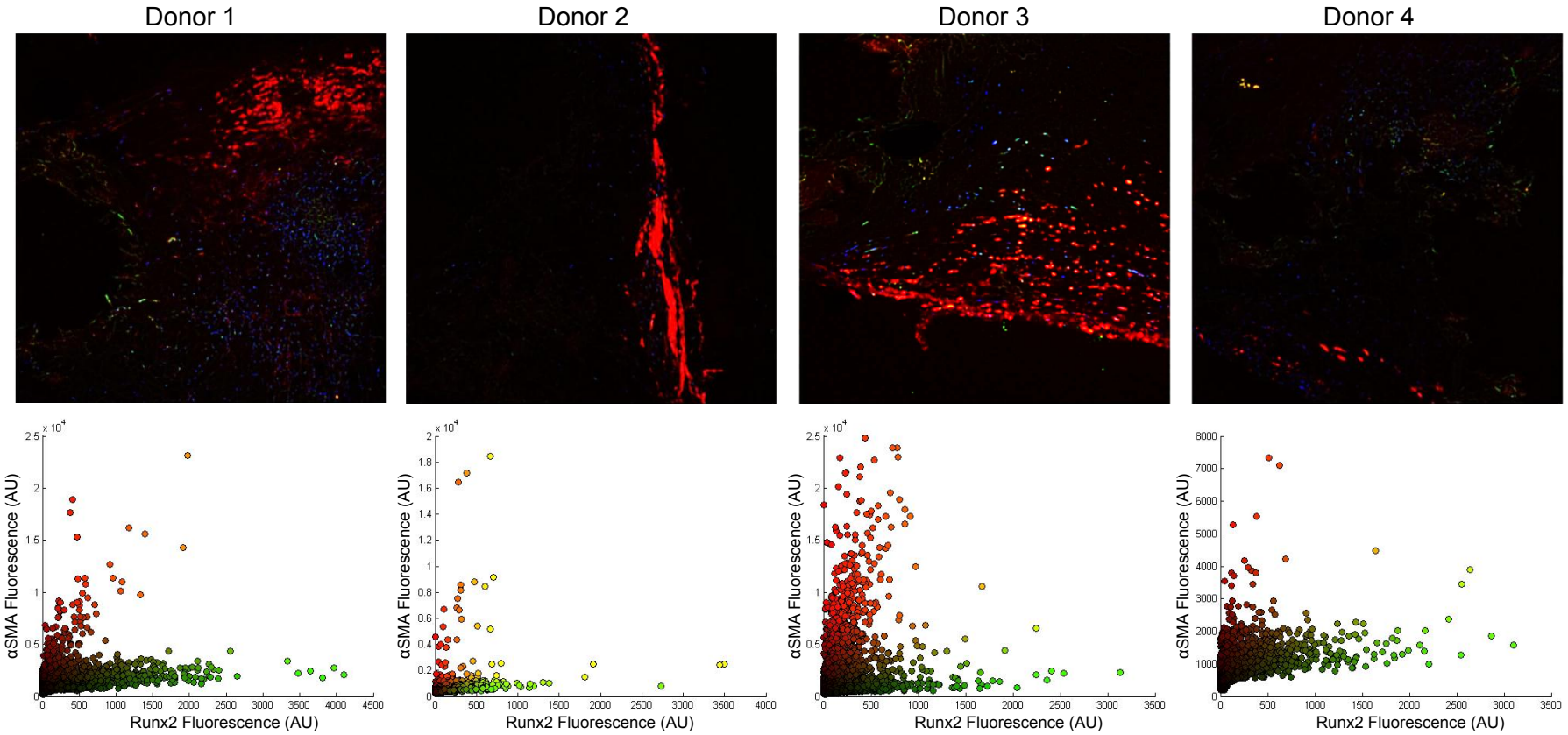

ALP Activity
ALP activity (AU/ug dsDNA)
20
18
16
14
12
10
8
6
4
2
0
*
*
*
*
CM
OM
OM + TNF α
SCR
SiRNA

Figure VIII



# Supplemental Figure IX



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