Supporting Information

A "Sandwich" Cell Culture Platform with NIR-responsive Dynamic Stiffness to Modulate Macrophage Phenotypes

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Synthesis of MA-HA

MA-HA was synthesized according to the method described in the previous work with sight change[1]. Briefly, 1 g hyaluronic acid (Mw=100 kDa) was dissolved in 100 ml deionized water, followed by adding 8 ml methacrylic anhydride solution dropwise. The pH value of the mixture was maintained 8~9 by adding 5M NaOH solution. The reaction mixture was kept in ice bath for another 24h. MA-HA was precipitated and collected by pouring the solution in 10-fold volume of pre-cold ethanol and centrifugation. The obtained raw MA-HA was then dialyzed (MWCO 3500 Da) against deionized water for 3 days, and the final product was obtained by lyophilization and stored at -20 °C. The purified MA-HA was characterized by ^{1}H NMR, from which the methacrylate degree of MA-HA was quantified. The grafting degrees of double bonds calculated from the results of ^{1}H NMR in Figure S1 were 150%.

The temperature-responsive stiffness-rise behavior of the "sandwich" platform

A cylindrical polystyrene mold (diameter: 20 mm, depth: 2 cm) was used to build the sandwich material platform for the study of temperature-responsive stiffness-rise behavior. The same volume ratio was used in this test. After adding 1000 μl calcium ion solution in the bottom, 600 μl PCM (premixed with n-tetradecol and paraffin at the ratio of 7: 3 under 60 ºC) was added on the preheated liquid level. After cooling, 1000 μl hydrogel was gelatin on the top. Kept at 4 ℃ for 3 hours, the soft hydrogel on the sandwich platform was built and the stiffer one was gotten by placing the plate in a 60 ℃ oven for 10 minutes. The influence of the concentration of calcium ion (0%,

5%, 10%, 20%) at the bottom and the mass ratio of sodium alginate (1%, 1.5%, 2%) in the stiffer hydrogel were studied separately by rheological test. The store and loss modulus were showed in Figure S2.

For infrared thermal images, the "sandwich" platform was built in a cylindrical glass cuvette (diameter: 10 mm, depth: 2 cm), and the same liquid volume ratio was used. The 10% Ca^{2+} Solution was stained in purple by pigment. The infrared thermal images showed in Figure S3 were taken by near infrared imager (Fotric, USA). Every picture was taken after 1.4 w/cm^2 808nm NIR illumination for 480s.

The mass swelling test of the hydrogel

The mass swelling test is conducted by immersing the hydrogel (W_0) into Milli-Q water for 24h in 37 ºC. Then the final mass was weighed (W_w). The swelling ratio was calculated according to the formular.

$$Q_v = \frac{V_Q}{W_0} = \frac{\frac{W_W - W_0}{\rho_Q}}{W_0} \times 100\% \quad (1)$$

The results were showed in Figure S4b.

Cells cultured on the static hydrogel with different modulus

The method of cell isolation was published in the previous research[2].

For previous cell morphological study, pure hydrogel was built on glass plate grafted with double bond to fix the hydrogel. The grafting method was mentioned in the pre-published article[3]. Silane coupler (G-570) was used to be the double bond grafting agent. After UV-ozone treatment, silane coupler (G-570) was added to the glass slide followed by keeping in 100 ºC oven for 30 mins and then 110 ºC for 10 mins. The

slide was washed and exposed to UV-light for 30 min. The pure soft hydrogel is built on the glass slides and the slides were loaded in the 24-wells plate. Then washed with 3 × PBS and immersed into 5 mg/ml RGD for 2 h at 37 ºC to build cell adhesion site. Three different stiffness hydrogels were made by immersing the slide with soft hydrogel into 0%, 0.5% and 10% calcium ion solution for 1 h at 37 ºC. After washed with 5 × PBS, the hydrogel was used for cell culture. The cell concentration of the static research is 150 000 cells per well.

The stiffness of the hydrogel was characterized by dynamic oscillatory time sweeps with the pure hydrogel preparation methods mentioned before. The statistical results were showed in Figure S6d.

The attached cell numbers were calculated by digesting off the hydrogel after incubation for 24 h. The statistical results were showed in Figure S6e.

To find out the morphological difference of cells cultured on different stiffness hydrogel, BMDMs were stained with calcein-AM (Beyotime, China) and observed under fluorescence microscope after culturing for 24 h. The pictures were showed in Figure S4.

The phonotype of the BMDMs cultured on different stiffness hydrogel was also observed by immunofluorescence. Following culture for 24 h, the cells were fixed with methanol for 15 min at 37 °C, washed 3× with PBS and permeabilized with 0.1% Triton X-100 for 10 min at 4 ºC. Then washed and blocked with 3% w/v bovine serum albumin (BSA, AMRESCO, USA) in PBS for 90 min at 37°C. The blocking solution was then removed and the samples washed with PBS. Then cells were stained

with fluorescein isothiocyanate-labeled inducible nitric oxide synthase (iNOS) antibody (1:50, BD Pharmingen, USA) and PE-labeled arginase-1 (Arg-1) antibody (1:50, Santa Cruz, USA), washed twice with PBS, counterstained with DAPI (1: 200, Invitrogen, USA), and observed under a fluorescence microscope (IX81, Olympus, Japan).

For further verify the difference between the BMDMs cultured on the soft and the rigid hydrogels, the cell morphology was tested by Confocal Laser Scanning Microscope (CLSM). BMDMs were seeded on the static soft and rigid hydrogel for 24 h and fixed with 4% PFA at 37 ºC for 10 min, then washed with PBS for 3 times and permeabilized at 4 ºC using 0.5% Triton X-100 (Sigma-Aldrich, USA) for 10 min. After being rinsed with PBS 3 times, the samples were incubated into 3% BSA / PBS for 90 min at 37 ºC. Mouse monoclonal vinculin primary antibody (dilution 1:100, Abcam, UK) and rhodamine-labeled phalloidin (dilution 1:100, Thermo Fisher Scientific, USA) were used for immunostaining, after incubating overnight at 4 ºC, the cell was washed thrice with PBS and incubated with FITC labeled goat-anti-mouse IgG (Boster, China) for 12 h at 4 ºC. Then BMDMs were stained with 4', 6'-diamidino-2-phenylindole (DAPI, dilution 1:200, Sigma-Aldrich, USA) at room temperature for 10 min. After being rinsed thrice in PBS, BMDMs were observed under CLSM.

The observation of the BMDM's morphology cultured on the dynamic sandwich hydrogel.

BMDMs were seeded on different sandwich hydrogel (Ca^{2+}+/–) for 24 h, after NIR

light illuminating (808 nm, LSR-PS-FA, Lasever Inc., China) for 480 s and culturing for another 24h, it was fixed with 4% PFA at 37 ºC for 10 min, then washed with PBS for 3 times and permeabilized at 4 ºC using 0.5% Triton X-100 (Sigma-Aldrich, USA) for 10 min. After being rinsed with PBS 3 times, the samples were incubated into 3% BSA / PBS for 90 min at 37 ºC. Mouse monoclonal vinculin primary antibody (dilution 1:100, Abcam, UK) and rhodamine-labeled phalloidin (dilution 1:100, Thermo Fisher Scientific, USA) were used for immunostaining, after incubating overnight at 4 ºC, the cell was washed thrice with PBS and incubated with FITC labeled goat-anti-mouse IgG (Boster, China) for 12 h at 4 ºC. Then BMDMs were stained with 4', 6'-diamidino-2-phenylindole (DAPI, dilution 1:200, Sigma-Aldrich, USA) at room temperature for 10 min. After being rinsed thrice in PBS, BMDMs were observed under CLSM.

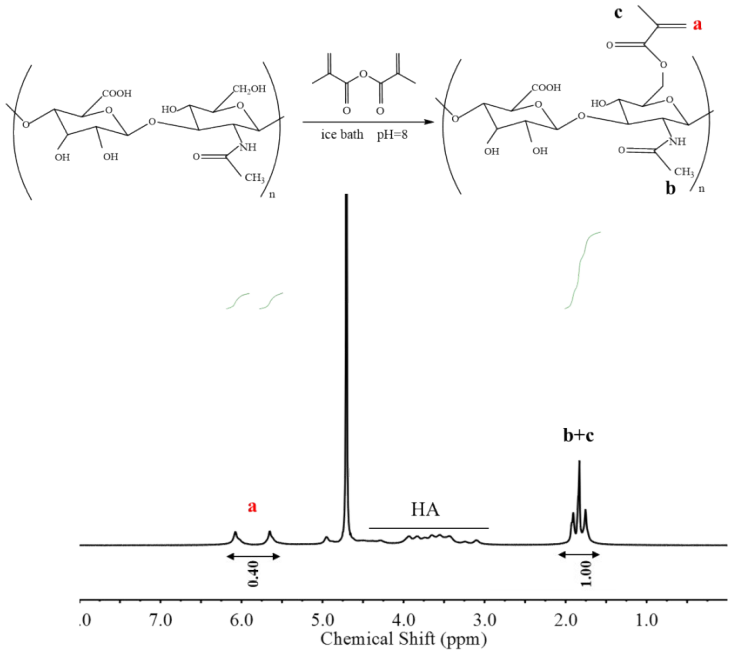

Figure S1. Synthesis route of methacrylate anhydride-modified hyaluronic acid (MA-HA) and its H^1 NMR spectrum.

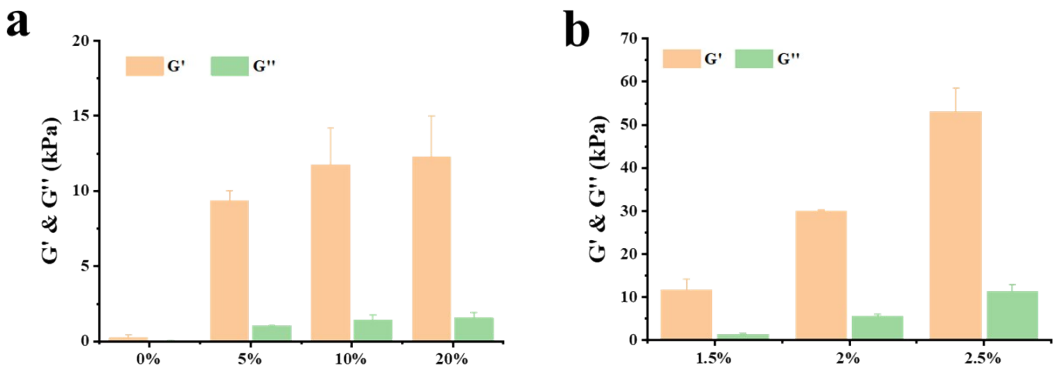

Figure S2. (a) The effects of the concentration of Ca^{2+} solution at the bottom of "sandwich" platform on the storage and loss modulus of the hydrogel, respectively. 1.5% mass ratio SA was used; (b) the effects of mass ratio of SA on the store and loss modulus of the hydrogel, respectively. 10% Ca^{2+} solution was used. The PCM was built by mixing n-tetradecol and paraffin wax at the ratio of 7: 3 without IR780. The liquid volume of the "sandwich" platform from the bottom to top was 1000, 600, 1000 μl respectively. The platform was heated at 60°C for 10 min.

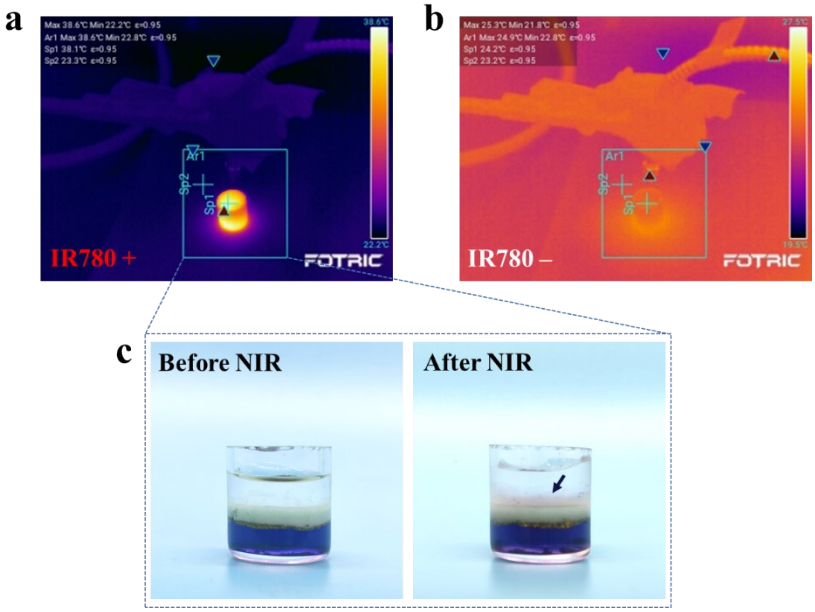

Figure S3. (a) the infrared thermal image of the platform with IR780 (noted as "IR780 +"). (b) the infrared thermal image of the platform without IR780 (noted as "IR780 –"). (c) the photographs of the "sandwich" platform with IR780 before and after NIR exposure were also displayed. Black arrow indicates the hydrogel layer with lower transparency.

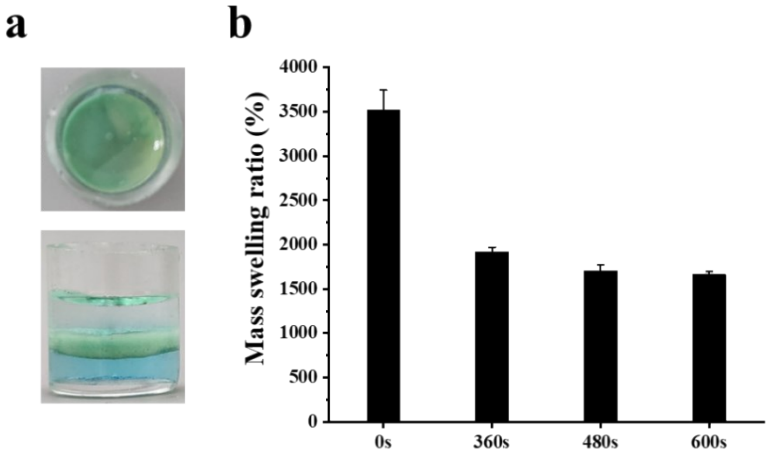

Figure S4. (a) The gross view of the "sandwich" cell culture platform. (b) the mass swelling ratio of the hydrogel layer in the platform with irradiation time.

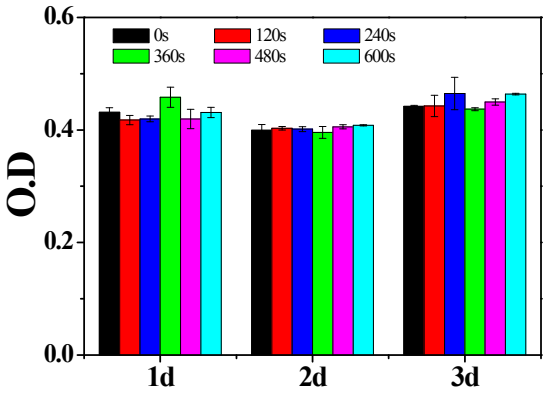

Figure S5. The viabilities of macrophages cultured on the "sandwich" platforms without Ca^{2+} and being irradiated by 1.5 w/cm^2 NIR for different time.

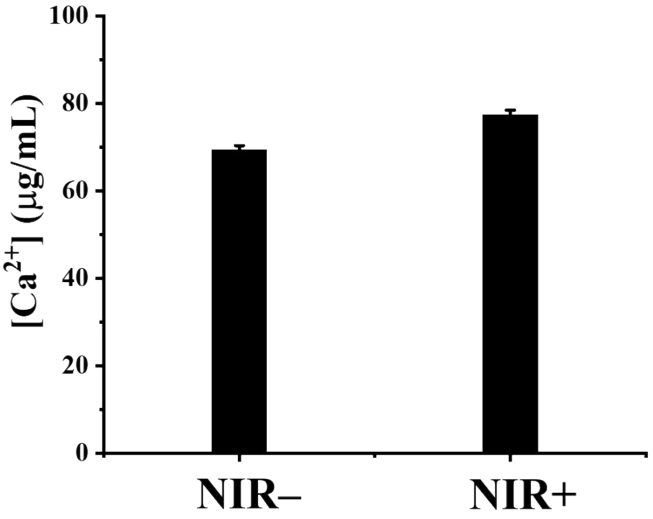

Figure S6. The concentrations of calcium ions in the culture mediums on the platform with/ without NIR.

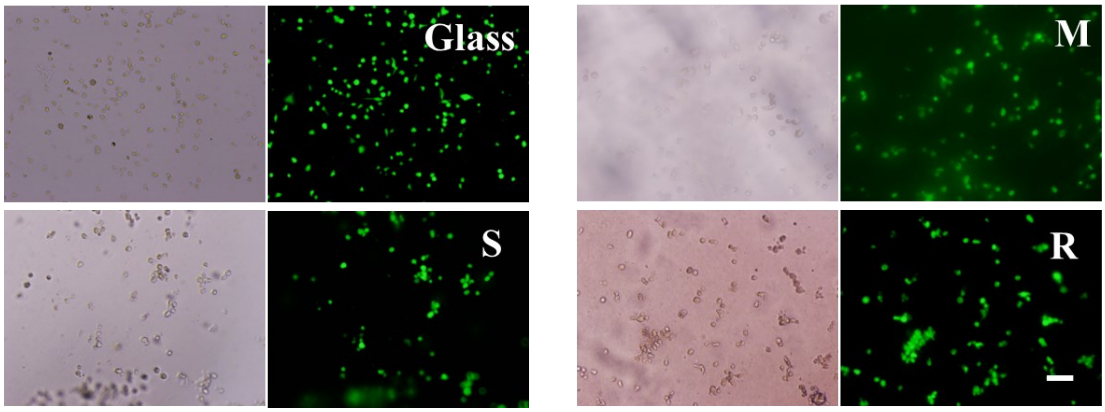

Figure S7. The bright field and fluorescent images of BMDMs (stained by calcein-AM) cultured on Glass, MA-HA&CA hydrogels with low (S), medium (M) and high (R) stiffness. Scale bar is 100 μm.

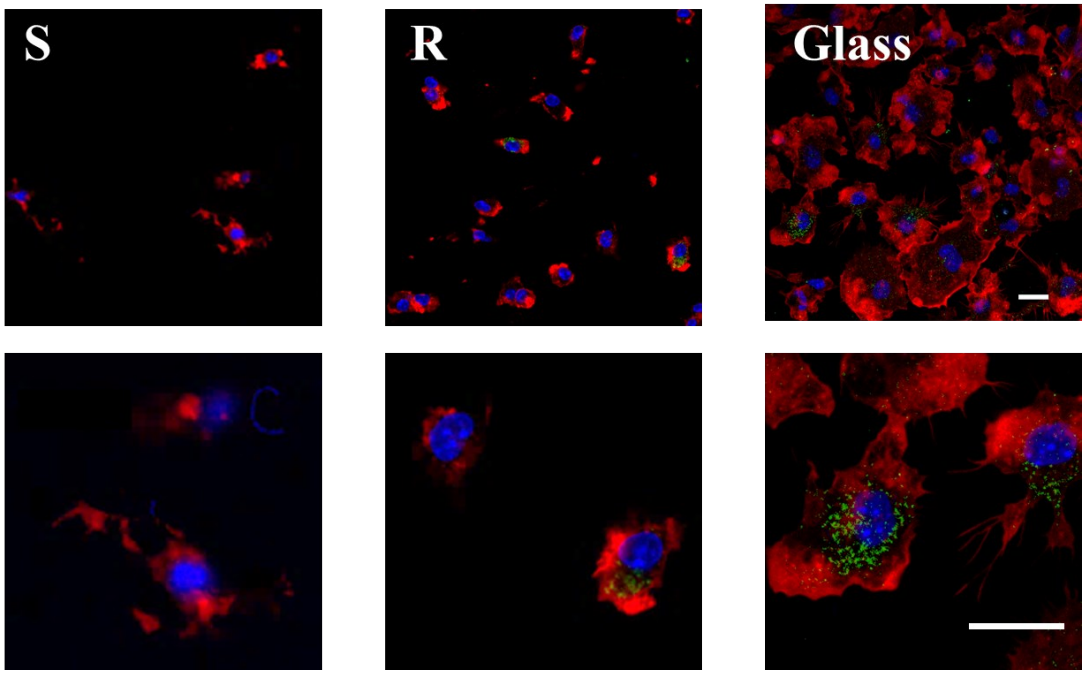

Figure S8. Fluorescent images of BMDMs immunostained for actin (red), nuclei (blue), and vinculin (green) by confocal laser scanning microscope (CLSM). The cells were cultured on pure soft (S), rigid (R) MA-HA&CA hydrogels and glass. bar:20um.

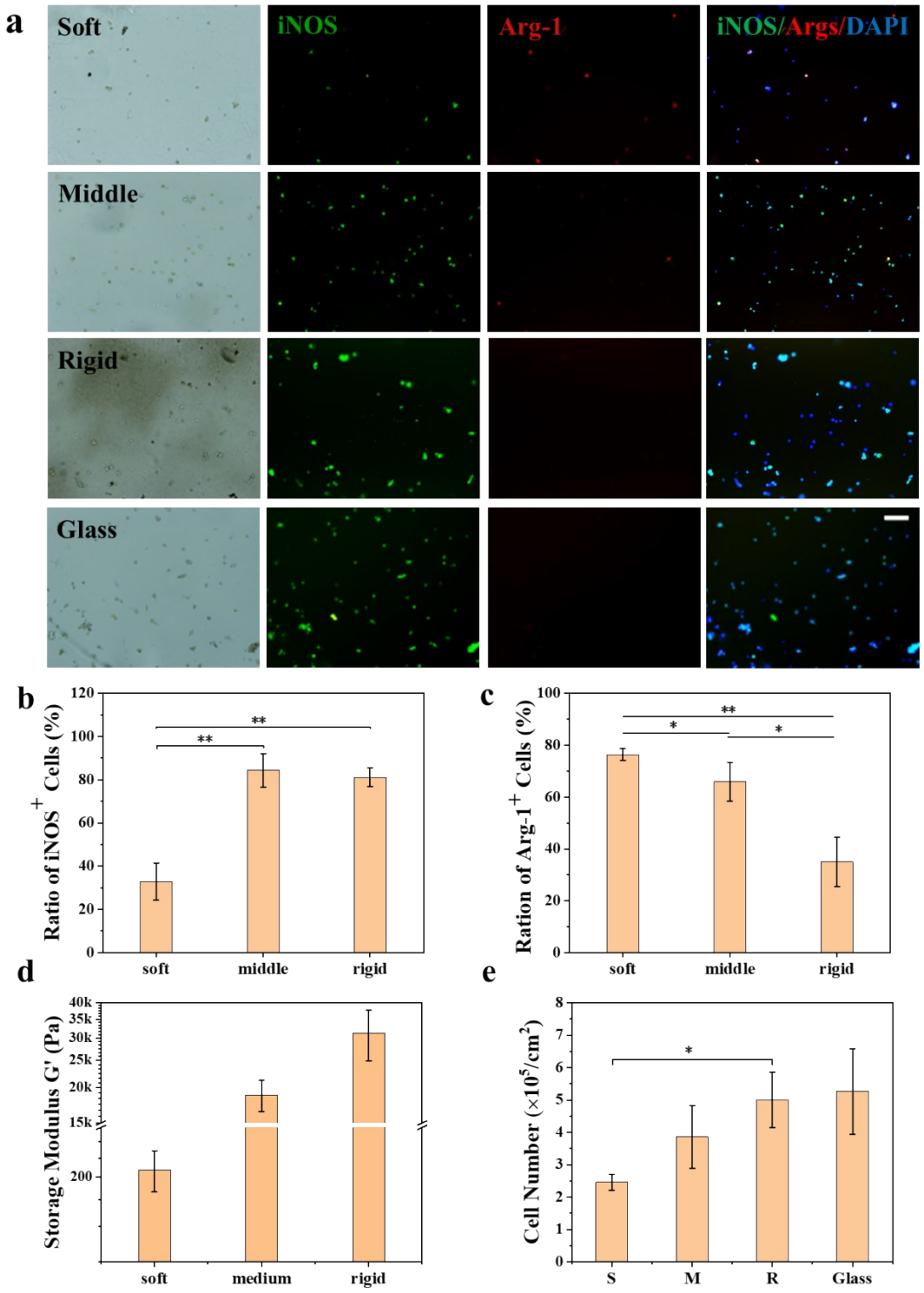

Figure S9. (a) The fluorescent images of BMDMs cultured on the MA-HA&CA hydrogels with different moduli immunostained for iNOS (M1 marker, green), arginase-1 (Arg-1, M2 marker, red), and nuclei (DAPI, blue) to verify the phonotype

of BMDMs on the different stiffness hydrogel. Scale bar is 100 μm. (b), (c) Relative expression levels of iNOS and Arg-1 per 50000 cells in vitro were quantified by ImageJ and normalized with the cell number. (d) the store modulus of the hydrogel used in the static immunostaining test verified by rheological test. (e) the cell number attached on the different modulus hydrogel. (* and ** indicate $p < 0.05$ and 0.01, respectively).

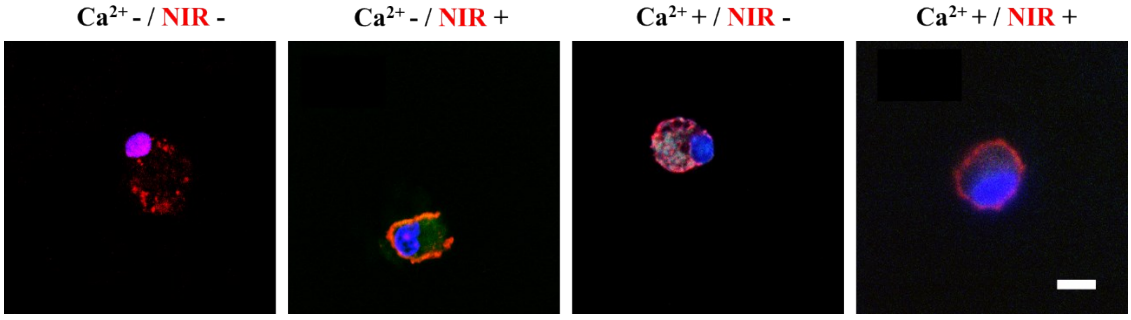

Figure S10. Fluorescent images of BMDMs immunostained for actin (red), nuclei (blue), and vinculin (green) measured by CLSM. The cells were cultured on different dynamic "sandwich" platforms. Indeed, "+/–" represent different "yes/no" condition or stimuli respectively (i.e. Ca^{2+}+ / NIR– indicate the platform was built with Ca^{2+} but without NIR exposure during cell culture).

Reference

[1] S.J. Huang, C.F. Wang, J.W. Xu, L. Ma, C.Y. Gao, In situ assembly of fibrinogen/hyaluronic acid hydrogel via knob-hole interaction for 3D cellular engineering, Bioactive Materials 2(4) (2017) 253-259.

[2] X.W. Zheng, L.B. Xin, Y.L. Luo, H. Yang, X.Y. Ye, Z.W. Mao, S.Y. Zhang, L. Ma, C.Y. Gao, Near-Infrared-Triggered Dynamic Surface Topography for Sequential Modulation of Macrophage Phenotypes, ACS Applied Materials & Interfaces 11(46) (2019) 43689-43697.

[3] M. Guvendiren, J.A. Burdick, Stiffening hydrogels to probe short- and long-term cellular responses to dynamic mechanics, Nature Communications 3 (2012) 792.