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Soft tissue-mimicking hydrogel stiffness modulates polarisation of human monocyte-derived macrophages

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Minimising the foreign body response (FBR) to implants remains an unmet clinical challenge. The mechanical properties of biomaterials, particularly stiffness, have been shown to influence macrophage activation and FBR. However, current literature on the effects of material stiffness on macrophage activation presents conflicting results. These inconsistencies may stem from the use of cell lines or murine primary cells, which do not fully represent the behaviour of primary human monocyte-derived macrophages. Additionally, many previous studies have focused on stiffness values that fall outside the physiological range of soft tissues. In this study, we investigated how variations in stiffness affect the activation status of human monocyte-derived macrophages using alginate methacrylate (ALMA) hydrogels. The hydrogel stiffness was tuned within a physiologically relevant range (0.25–4.5 kPa) to mimic the mechanical properties of soft tissues. We found that increasing hydrogel stiffness consistently upregulated pro-inflammatory markers. Specifically, the stiffest hydrogel (ALMA 6% w/v) induced higher secretion of TNF- α and increased the calprotectin-to-mannose surface marker ratio, both hallmarks of inflammatory macrophages. Moreover, macrophages cultured on stiffer hydrogels exhibited a more elongated morphology and greater spreading. These findings provide new insight into how small changes in stiffness, within a soft tissue-relevant range, can modulate the inflammatory behaviour of human macrophages. Our results, together with findings from the literature, suggest that the contradictory data on the effects of stiffness on macrophages may be attributed to other factors, such as viscoelasticity, surface chemistry, and protein absorption, which warrant further investigation.

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Introduction

A recurrent adverse phenomenon following biomaterial implantation is the foreign body reaction (FBR), which often leads to the formation of a fibrous capsule surrounding the implant. This response can compromise implant functionality and therapeutic efficacy.^{1–4} Macrophages play a critical role in the development of FBR. Studies using immune cell knockouts in animal models have identified macrophages as the primary drivers of fibrous capsule formation.⁵ Macrophages are phagocytic cells⁶ that can be broadly classified as either tissue-resident or monocyte-derived. Tissue-resident macrophages are a heterogeneous population, such as Kupffer cells, Langerhans

cells, and microglia, that act as sentinels within their respective tissues.⁷ On the other hand, monocyte-derived macrophages differentiate locally following monocytes migration to sites of injury or infection. Although macrophages have traditionally been classified as either classically-activated (M1) or alternatively-activated (M2), it is now recognised that they exist along a continuum of activation states,⁸ with M1 and M2 representing the prototypical pro- and anti-inflammatory extremes of this spectrum.⁹ An important feature of macrophages is their plasticity, the ability to dynamically shift phenotypes in response to environmental cues.¹⁰

It is known that the mechanical properties of biomaterials, including stiffness, affect macrophage polarisation.^{11,12} Several studies have reported that stiffer substrates promote M1 polarisation. For example, in a study using RAW 264.7 cells, a mouse macrophage cell line, M1 polarisation was significantly reduced on the polyethylene glycol (PEG) hydrogel with a lower modulus (130 kPa), compared to stiffer hydrogels (240 kPa and 840 kPa). Additionally, when these gels were tested *in vivo*, the softest hydrogel resulted in the thinnest layer of macrophages at the implant site.¹³ Similarly, a study using polyacrylamide

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The mechanical properties of the hydrogels were determined using rheological testing. Monocytes were isolated from healthy blood donors and cultured on ALMA hydrogels for 6 days. Macrophage phenotype was assessed using a combination of techniques, including morphological evaluation, surface marker expression and cytokine profiling. We observed that increasing hydrogel stiffness promoted a pro-inflammatory macrophage phenotype, as evidenced by elevated TNF- α secretion, an increased calprotectin-to-mannose receptor ratio, and a more elongated cell morphology.

Materials and methods

Materials

Alginate methacrylate (degree of methacrylation: 20%–40%), 2-hydroxy-4'-(2-hydroxyethoxy)-2-methylpropiophenone (Irgacure D-2959), 3-(trimethoxysilyl)propyl methacrylate, toluene, PBS, Tween20, RPMI-1640, fetal bovine serum (FBS), penicillin–streptomycin, L-glutamine, glycine, trypan blue solution, Macrophage Colony-Stimulating Factor (M-CSF), Granulocyte-Macrophage

Colony-Stimulating Factor (GM-CSF), Interferon- γ (IFN- γ), human recombinant IL-4, and goat serum were purchased from Sigma Aldrich. Sulfuric acid 1 N, anti-human calprotectin mouse immunoglobulin G1, TMB substrate kit, and LIVE/DEAD viability kit, Pierce™ chromogenic endotoxin quant kit were supplied by Thermo Scientific, UK. ToxiLight™ non-destructive cytotoxicity BioAssay kit and 100% lysis control set were purchased from Lonza. Buffy coats were obtained from healthy donors (National Blood Service, Sheffield, UK) after obtaining informed written consent and following ethics committee approval (Research Ethics Committee, Faculty of Medicine and Health Sciences, University of Nottingham). Ficoll-Plaque PREMIUM sterile solution was purchased by cytiva. LS columns, pre-separation filters (30 mm), and CD14 MicroBeads UltraPure human were bought from Miltenyi Biotec. Anti-human CD206 (MR) rabbit IgG1 primary antibody was provided by Abcam. Phalloidin-iFluor 594 was supplied by AAT Bioquest, Stratech. 4,6-Diamidino-2-phenylindole (DAPI), UltraPure EDTA (0.5 M, pH 8), AlexaFluor 488 goat anti-mouse IgG (H + L), and Texas Red-X goat anti-rabbit IgG (H + L) were purchased from Invitrogen. DuoSet® ELISA kits (TNF- α , IL-6, IL-10, CCL18) were purchased from Biotechne®/R&D systems.

Coverslips silanisation

Coverslips were functionalised as previously described with some modifications.⁴² Plasma cleaning (O₂ 100%, 10 min) was performed on glass coverslips ($d = 16$ mm), which were subsequently silanised with 3-(trimethoxysilyl)propyl methacrylate (2% v/v) in dry toluene for 24 h. The reaction was carried out under argon atmosphere. Coverslips were washed with fresh toluene ($\times 2$), acetone ($\times 3$), and dried under vacuum for 7 days prior to use.

Preparation of ALMA hydrogels

To fabricate ALMA hydrogel, Irgacure D-2959 aqueous solution (0.5% w/v) was prepared and subsequently added to alginate methacrylate powder. ALMA solutions (2%, 4%, and 6% w/v) were stirred overnight and underwent photo-crosslinking with UV light (365 nm) for 10 minutes. Samples that were used for the rheological characterisation were prepared as discs (2 mm, $r = 1.06$ cm²). Samples that were used for cell culture were prepared as thin film on silanised coverslips by creating a “sandwich” system with glass slides. UV-sterilization (260 nm) was performed for 40 minutes and samples were then left in penicillin-streptomycin (10 $\times$) overnight. Samples were washed 5 times with sterile PBS and incubated with RPMI-1640 (FBS 10% v/v, penicillin-streptomycin 1% v/v, L-glutamine 1% v/v) for 24 hours prior cell seeding.

In vitro hydrogel swelling

Hydrogels were immersed in 15 mL PBS at 37 °C and weighed multiple times over a 6-day period. The swollen weight was expressed as a percentage increase from the initial hydrogel weight, recorded immediately after UV photo-crosslinking.

Environmental scanning electron microscopy (ESEM)

Hydrogels were prepared and left equilibrating in PBS for at least 24 h prior the analysis. Samples were placed in a FEI Quanta 650 ESEM and images were captured using an optimised working distance for each sample at 5–10 kV, while humidity was kept between 88.9–96.3%.

Optical profilometry

Surface roughness of hydrated ALMA hydrogels was measured using an optical profilometer. Gels were equilibrated in PBS, blotted to remove excess liquid, and mounted on the stage. Scans were acquired with a 50 $\times$ objective over a 340 $\times$ 261 μ m² area, with 3 regions per sample to account for heterogeneity. Arithmetical mean height (S_a) and root mean square height (S_q) were extracted from topography maps using the instrument's analysis software (Zeta 3D Analysis).

Mechanical characterisation

Rheological measurements were performed with Anton-Paar rheometer, equipped with a 25 N load cell. Discs of ALMA hydrogels were prepared ahead the measurements and incubated in PBS until they reach the equilibrium swelling. Amplitude sweeps was carried out at a constant frequency of 1 Hz, between 1% and 1000% of shear strain. Frequency sweep was conducted within the linear viscoelastic (LVE) range, at a constant shear strain of 5%; storage and loss moduli were extrapolated at frequency 1 Hz. Stress relaxation was performed within the LVE range, at a constant shear strain of 5%; relaxation modulus was recorded for 200 s and expressed as a percentage decrease from the initial relaxation modulus.

Mesh size measurement

Rubber elasticity theory was used to calculate mesh size of ALMA hydrogels. The crosslinking density ρ_x (mol m⁻³) is determined from the storage modulus (G' , Pa), the universal gas constant (R , J mol⁻¹ K⁻¹), and the absolute temperature (T , K), as shown in

$$\rho_x = \frac{G'}{RT}. \quad (1)$$

The mesh size ξ (m) is then calculated from the crosslinking density (ρ_x) and Avogadro's number N_A , according to eqn (2).⁴³

$$\xi = \sqrt[3]{\frac{6}{\pi \rho_x N_A}} \quad (2)$$

X-Ray photoelectron spectroscopy

All samples were mounted onto stainless steel stubs using carbon sticky tabs. X-ray Photoelectron Spectroscopy (XPS) was conducted using a Kratos Axis Ultra DLD with a monochromatic Al X-ray source (1486.6 eV, 15 kV, 10 mA). Wide and high-resolution scans were conducted in addition to the measurement C 1s for calibration: charge corrected to 284.8 eV. Parameters for wide scan acquisition were as follows: 160



eV of pass energy, step size of 0.5 eV, and 1200 s of scan time. Parameters for high resolution scan acquisition were as follows: 20 eV of pass energy, step size of 0.1 eV, and 1200 s of scan time. Binding energies were measured over a range of 0–1300 eV. All spectra were analysed in CasaXPS constraining the Full Width at Half Maximum (FWHM) to the same value for all deconvoluted spectral peaks for the same element.

Monocyte isolation and differentiation

Buffy coats from healthy donors were obtained from the National Blood Service (Sheffield, UK). All experiments were conducted in compliance with the Declaration of Helsinki and the Human Tissue Act (2004), following ethics committee approval (2009/D055 Research Ethics Committee, Faculty of Medicine and Health Sciences, University of Nottingham). Informed consent was obtained from all donors prior to sample collection. Monocytes were isolated through positive selection (CD14 magnetic beads), using MACS magnetic cell separation system. Isolated monocytes were prepared to a cell density of 1×10^6 cell per mL in RPMI-1640 medium (10% FBS, 1% L-glutamine, 1% streptomycin and penicillin, 10 ng mL⁻¹ M-CSF) and seeded on ALMA hydrogel. To ensure cells were only seeded on the hydrogels, non-cultured-treated plates were used, and media was changed after 24 h to remove floating monocytes. Cells were incubated in a humidified incubator maintained at 37 °C and 5% CO₂ for 6 days (37 °C, 5% CO₂) until differentiation was completed; fresh media was added on day 3. Media was supplemented with M-CSF (10 ng mL⁻¹) on day 1 and 3 to induce the differentiation into naïve macrophages. As a reference, monocytes were also cultured on tissue culture plastic at the same cell density and conditions.⁴⁴

Live/dead viability assay

On day 6, media was removed, and macrophages were incubated with PBS containing a mixture of green-fluorescent calcein-AM (1:2000) and red-fluorescent ethidium homodimer-1 (1:500) for 30 minutes at 37 °C and 5% CO₂. Cells were imaged using an Olympus IX51 fluorescence microscope, with 10× objective.

Cytotoxicity measurement by using the ToxiLight assay

20 µL of culture supernatant was collected and added to a white assay plate. 100 µL of adenylate kinase detection reagent was added and incubated at room temperature (RT) for 5 minutes. Luminescence was read using a plate reader (Promega GloMax® explorer). A negative (M0 macrophages cultured on TCP) and positive (M0 macrophages cultured on TCP and lysed after 6 days of culture using the Toxilight™ 100% lysis reagent) were included.

Endotoxin quantification assay

ALMA 6% hydrogels were incubated with PBS (1.5 mL) in humidified incubator maintained at 37 °C and 5% CO₂ for 6 days (37 °C, 5% CO₂) to simulate cell culture conditions. Endotoxin standard solutions (0.1–0.01 EU per mL) and samples (50 µL) were added to 96-well plate and incubated

with amoebocyte lysate solution (50 µL) for 25 minutes at 37 °C. Chromogenic substrate solution (100 mL) was added and incubated for 6 minutes at 37 °C. Acetic acid 25% v/v (50 µL) was added as stop solution and absorbance was read at 405 nm using a plate reader (Promega GloMax® explorer). The endotoxin concentration in each condition was calculated using a 4-point standard curve.

Immunostaining of macrophages

Macrophages were fixed by incubation in paraformaldehyde 4% v/v (in PBS) for 15 min on day 1, day 3, and day 6. On day 1 and 3, cells were washed 3 times with 0.2% Tween-20 in PBS (0.2% v/v) followed by a blocking step with BSA (3% w/v) and glycine (1%) in PBS. Cells were washed 3 times with 0.2% Tween-20 in PBS and incubated with Phalloidin-iFluor 647 (1:1000) and DAPI (1:5000) in PBS (BSA 1% w/v) for 1 hour at RT. On day 6, cells were washed 3 times with 0.2% Tween-20 in PBS and two blocking steps were subsequently performed, firstly with BSA (3% w/v) and glycine (1%) in PBS, then with goat serum (5%) in PBS. Macrophages were washed 3 times with 0.2% Tween-20 in PBS and incubated with anti-human calprotectin mouse immunoglobulin G1 (IgG1) antibody (2 mg mL⁻¹) and anti-human CD206 (MR) rabbit IgG1 primary antibody (1 mg mL⁻¹) overnight at 4 °C. Cells were washed 3 times with 0.2% Tween-20 in PBS and incubated with AlexaFluor-488 goat anti-mouse IgG (8 mg mL⁻¹) and Rhodamine Red goat anti-rabbit IgG secondary antibody (8 mg mL⁻¹) for 1 hour at RT. Cells were further incubated with Phalloidin-iFluor 594 (1:1000) and DAPI (1:5000) in PBS (BSA 1% w/v) for 1 hour at RT. Cells were washed 3 times with PBS and imaged using a Leica TCS SPE confocal microscope.⁴⁵ Image analysis was performed using CellProfiler software (<https://www.cellprofiler.org/>) to quantify the cell number, the eccentricity, the cell area, and the fluorescent signal corresponding to calprotectin and mannose receptor markers.

Enzyme-linked immunosorbent assay (ELISA)

For TNF-α, IL-6, IL-10, and CCL18, 384-plates were coated with 25 µL capture antibody (TNF-α: 4 µg mL⁻¹, IL-6, IL-10, and CCL18: 2 µg mL⁻¹) overnight on a rocker at RT. ELISA plates were washed 3 times with PBS containing 0.05% tween-20 (100 µL) and blocked with 75 µL BSA 1% w/v for 1 hour at RT. Supernatant was thawed and brought to RT. Plates were washed 3 times with PBS containing 0.05% tween-20 (100 µL) and incubated with 25 µL supernatant and standard solutions (TNF-α: 1000–15.6 pg mL⁻¹, IL-6: 600–9.38 pg mL⁻¹, IL-10: 2000–31.2 pg mL⁻¹, and CCL18: 500–7.81 pg mL⁻¹) for 2 hours on a rocker at RT. Plates were washed 3 times with PBS containing 0.05% tween-20 (100 µL) and incubated with 25 µL detection antibody (TNF-α, IL-6, and IL-10: 50 ng mL⁻¹, CCL18: 100 ng mL⁻¹) for 2 hours on a rocker at RT. Plates were washed 3 times with PBS containing 0.05% tween-20 (100 µL) and incubated with 25 µL streptavidin-HRP conjugate (40-fold diluted) for 20 minutes while protected from light on a rocker at RT. Plates were washed 3 times with PBS containing 0.05% tween-20 (100 µL) and incubated with 25 µL TMB substrate for



20 minutes while protected from light on a rocker at RT. Sulfuric acid 1 N (12.5 μ L) was added as stop solution and absorbance was read at 450 nm and 570 nm using a plate reader (Promega GloMax® explorer). The cytokine concentration in each condition was calculated using a 7-point standard curve.

Statistical analysis

Hydrogel characterisation data is presented as mean $\pm$ standard deviation (SD) with each experimental condition tested in triplicate. Mechanical property and endotoxin data is presented as mean $\pm$ standard deviation of three experimental repeats ($n = 3$) per condition. Cell culture data is presented as mean $\pm$ standard error of mean (SEM) from three independent experiments ($N = 3$), each with three experimental repeats ($n = 3$) per condition. Statistical analysis was carried out in GraphPad PRISM 9.0. One-way ANOVA was performed to determine significant differences between experimental conditions ($\alpha = 0.05$). Tukey's *post hoc* test was performed for pair-wise comparisons. Pairs with significant differences were labelled as $*p \leq 0.05$, $**p < 0.01$, $***p < 0.001$ and $****p < 0.0001$.

Results & discussion

In vitro swelling (Fig. 1B) showed that all three hydrogel compositions reached equilibrium in less than 24 hours, with ALMA 4% and 6% exhibiting a similar weight increase of 20%. The composition with 2% methacrylate alginate showed a higher swelling, reflecting the lower concentration of the polymer involved in forming the 3D network. To quantitatively determine the mesh size, amplitude sweeps (Fig. S1) were performed, and the rubber elasticity theory was applied to relate the storage modulus (G') to the network crosslinking density.^{43,46,47} Increasing ALMA concentration led to significantly smaller mesh sizes, showing an inverse correlation with stiffness (Fig. 1C). This observation is consistent with the fact that higher polymer concentrations yield stiffer hydrogels by forming denser crosslinked networks, which reduces both void space and the swelling capacity.⁴⁸ Environmental scanning electron microscopy (ESEM) images (Fig. 1D) were collected to characterise the surface of hydrogels at different ALMA concentrations in their native hydrated state. To complement ESEM observations, we further characterised surface topography using optical profilometry. Profilometry of hydrated gels provided quantitative roughness parameters, including the arithmetical mean height (S_a) and root mean square height (S_q). Roughness was higher for ALMA 2% ($S_a = 41.6$ nm; $S_q = 53.4$ nm) compared with ALMA 4% ($S_a = 26.2$ nm; $S_q = 33.4$ nm) and 6% ($S_a = 24.3$ nm; $S_q = 30.7$ nm), while no significant differences were observed between the 4% and 6% hydrogels (Fig. S2A and S2B). Both ESEM and profilometry indicated that surface smoothness increased with polymer concentration.

Protein adsorption and surface chemistry were assessed by XPS (Fig. 1E). Analysis of the integrated N 1s peaks, which

arise exclusively from adsorbed proteins as ALMA lacks nitrogen, showed no significant differences in nitrogen content among the compositions (Fig. 1F), indicating comparable protein adsorption across all formulations. Similarly, the quantification of C 1s (Fig. 1G) and O 1s (Fig. 1H) peaks revealed no detectable changes in surface functional groups with increasing polymer concentration. It is important to note that XPS provides only elemental composition and relative abundance of elements and cannot determine the identity, conformation, or orientation of the adsorbed proteins. Complementary approaches, such as mass spectrometry, would be required to fully characterise the adsorbed protein layer.

The mechanical properties of ALMA hydrogels were determined using rheological assessments after reaching equilibrium swelling. Frequency sweeps (Fig. 2A) were carried out within the LVE, previously determined through amplitude sweeps, to quantify the storage (G') and loss (G'') moduli (Fig. 2B), and the complex viscosities (Fig. 2C) of the ALMA hydrogels. While ALMA 2% and 4% did not show a significant difference in their G' (257.9 Pa and 1195.7 Pa, respectively) and G'' values (33.8 Pa and 35.2 Pa, respectively), the ALMA 6% hydrogel exhibited significantly higher moduli (G' value of 4426.6 Pa and G'' value of 175.3 Pa) compared to the two softer hydrogels. Together, these three compositions span stiffness values across the physiologically relevant range of soft tissues – from very soft tissues, such as the lens (0.2–0.8 kPa), to intermediate tissues, including the thyroid (1.3 kPa), pancreas (1.1 kPa), lung (1.5 kPa), adipose tissue (1.6 kPa), liver (2 kPa), and brain (1 kPa), and to relatively stiffer organs such as the kidney (4–5 kPa)³² – thus enabling investigation of cell responses across a representative mechanical spectrum. Stress relaxation was also investigated as many studies have shown the effect of viscoelasticity on cell responses.^{49–51} All three hydrogels showed similar stress relaxation profiles (Fig. 2D), confirming that observed differences in macrophage activation status were due to stiffness rather than viscoelasticity.

To provide an overview of the experimental strategy used to investigate macrophage responses to hydrogel stiffness, a schematic representation is shown in Fig. 3.

A live/dead assay (Fig. 4A) was performed to assess the toxicity of ALMA hydrogels. The data showed that macrophages remained viable after 6 days of culture across all hydrogels, suggesting comparable biocompatibility. The high cell viability was also supported by the ToxiLight assay which showed no significant differences in metabolic activity between macrophages cultured on the hydrogels and those cultured on TCP controls (Fig. 4B). Together, these results demonstrated the suitability of ALMA hydrogels as biocompatible substrates for macrophage differentiation and culture.

The expression levels of calprotectin (pro-inflammation marker) and mannose receptor (anti-inflammatory markers) were quantified by immunofluorescent staining and image analysis, in line with our previous works⁵² (Fig. 5A). To ensure cross-sample comparisons, the number of imaged cells was quantified across the different hydrogels. No significant differ-



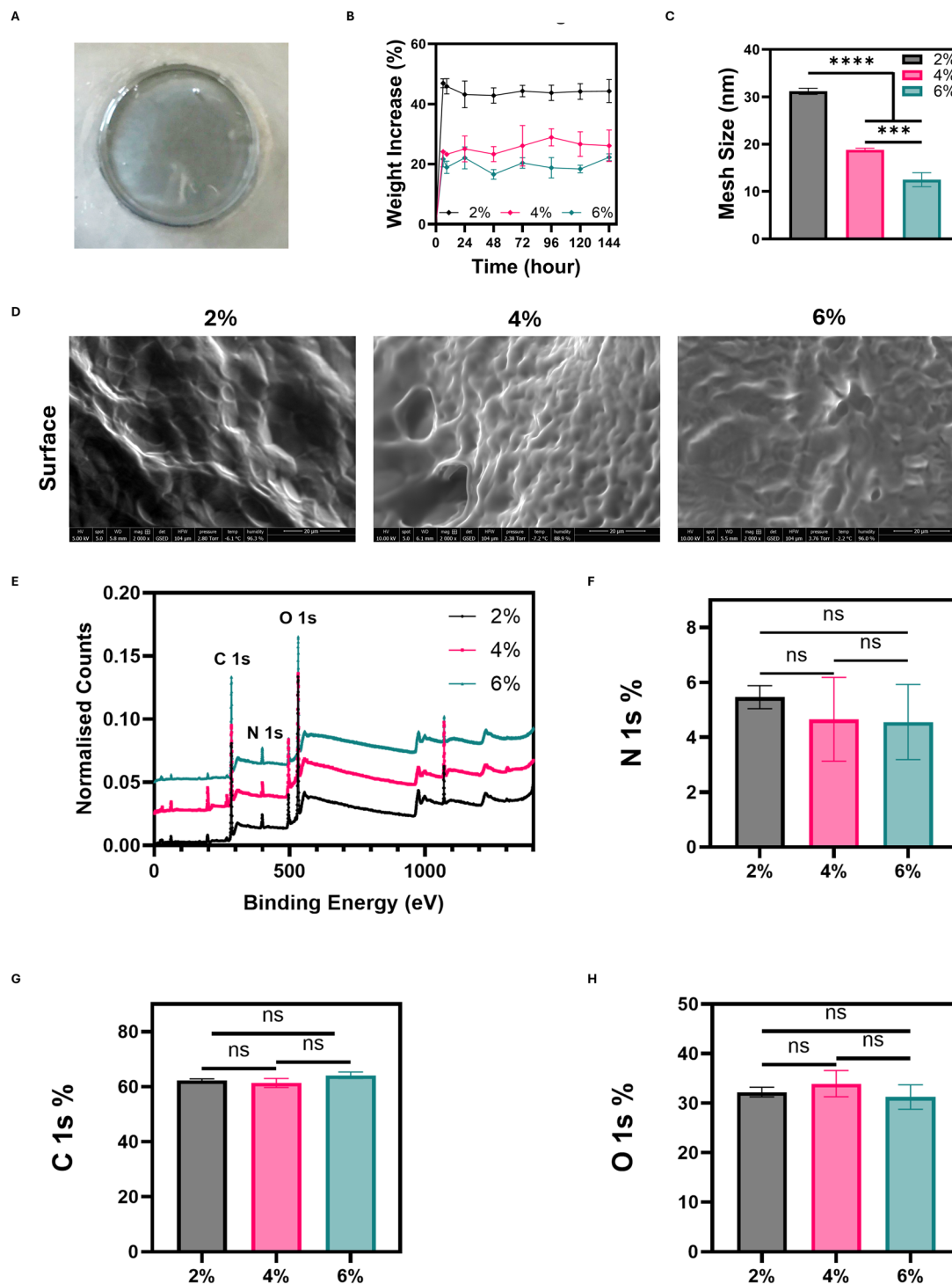


Fig. 1 Swelling, mesh size, surface morphology and surface adsorbed proteins. (A) Gross view of an ALMA hydrogel disc. (B) Swelling equilibrium and (C) mesh size of ALMA hydrogels at 2%, 4%, and 6% w/v calculated by using the rheological data and the rubber elasticity theory. (D) Representative environmental scanning electron microscopy (ESEM) images of the hydrogel surfaces. (E) XPS wide-scan spectra of ALMA hydrogels following 24-hour incubation in serum-containing culture medium. Quantification of (F) N 1s, (G) C 1s, and (H) O 1s peaks.

ences in cell number were detected (Fig. 5B). The calprotectin-to-mannose expression ratio was used as a surrogate for assessing the macrophage activation status (Fig. 5C). There was no significant difference in the calprotectin-to-MR ratio between

macrophages cultured on ALMA 2% and 4%. Macrophages cultured on 2% and 4% ALMA expressed more MR relative to calprotectin as the ratios were below one. In contrast, calprotectin was expressed significantly more (*ca.* 6-fold) in macrophages



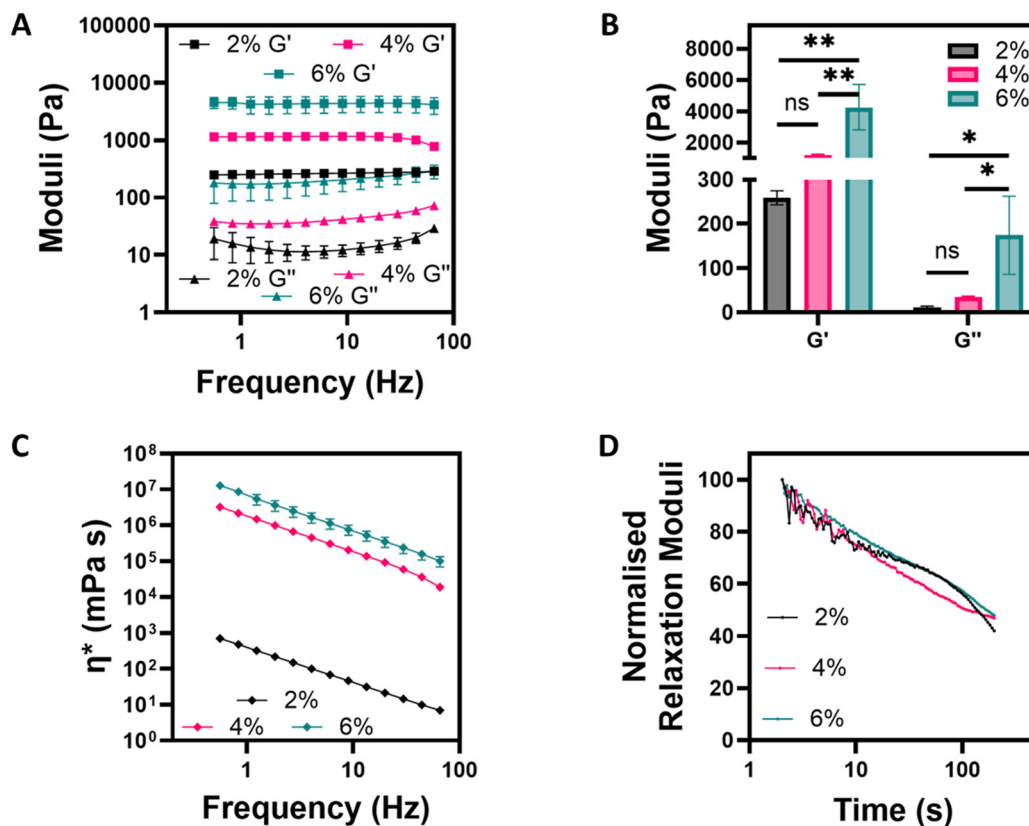


Fig. 2 Rheological characterisation of methacrylate alginate. (A) Frequency sweeps, (B) storage (G') and loss (G'') moduli, (C) complex viscosity, and (D) normalised stress relaxation of ALMA hydrogels. Frequency sweeps and stress relaxation were performed within LVE region (5% strain).

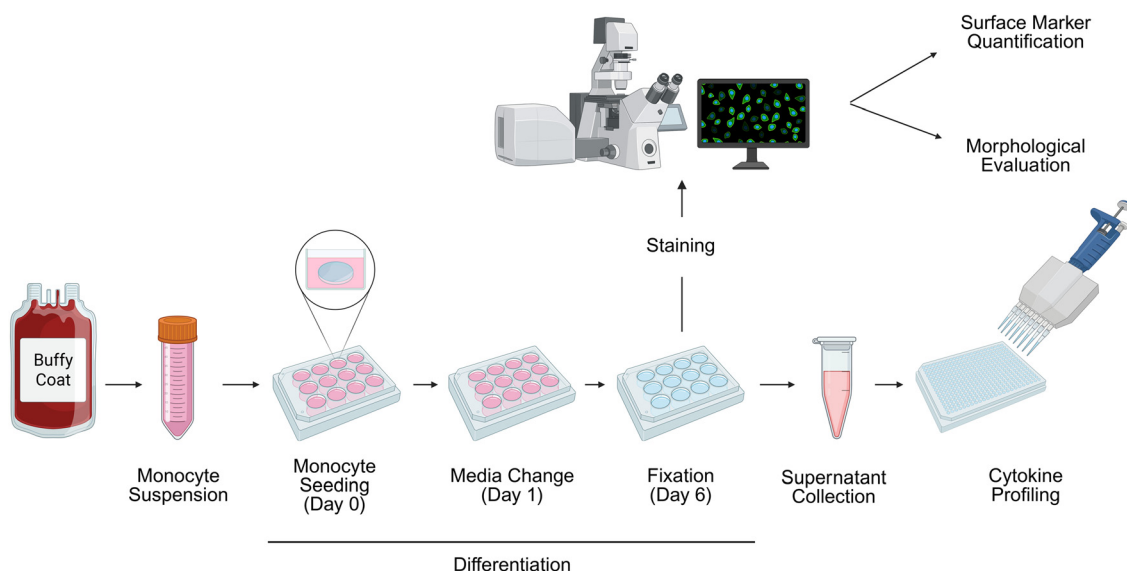
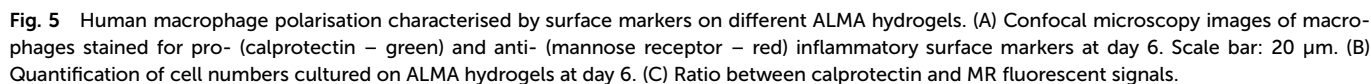
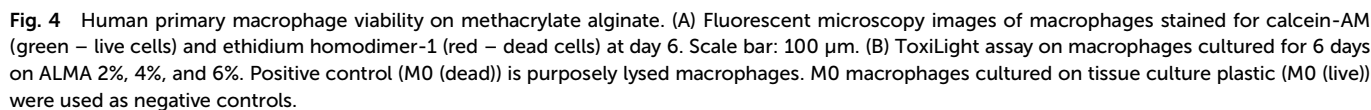


Fig. 3 Overview of the experimental workflow. Monocyte isolation from healthy blood donor, differentiation and characterisation of morphology, surface markers and cytokine profiling of macrophages.

that were cultured on ALMA 6%. This suggested that a significant increase in the storage modulus (G') promoted a shift towards a more pro-inflammatory, M1-like phenotype.

To further characterise macrophage activation in response to different stiffnesses, monocytes were stained for nuclei and F-actin to monitor their morphology over the 6-day culture





a rounder shape. To quantitatively compare cell morphologies, parameters including cell eccentricity and area were measured. Eccentricity was used to quantify cell elongation, where a value

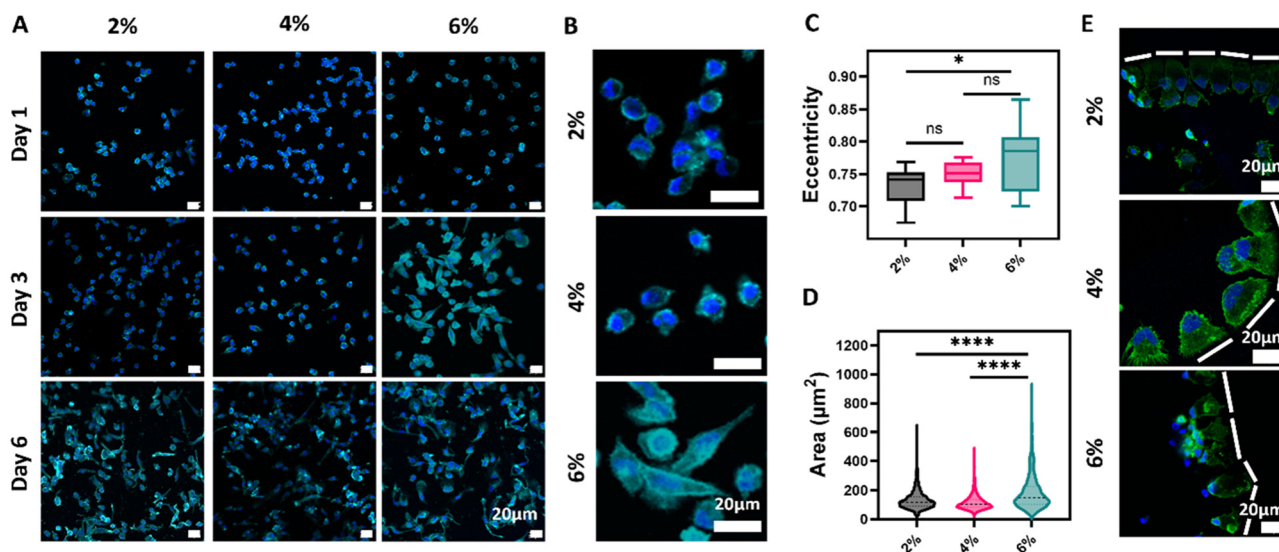


Fig. 6 Human macrophage morphology on different alginates. (A) Confocal microscopy images of monocyte (day 1 and 3) and macrophages (day 6) stained for nuclei (DAPI – blue) and F-actin (Phalloidin – cyan) at day 1, day 3, and day 6. Scale bar: 20 μm. (B) Zoomed-in confocal microscopy images representative of macrophage morphology at day 3. Scale bar: 20 μm. (C) Quantification of eccentricity and (D) area of macrophages at day 6. (E) Representative morphologies of macrophages adhered close to the edge of the disk-shaped ALMA hydrogels. White dashed lines represent the edges. Scale bar: 20 μm.

of zero corresponds to a perfect circle and one to a line. On day 6, macrophages on the stiffest ALMA hydrogel (ALMA 6%) displayed significantly higher eccentricity (~ 0.8) compared to those on the softest hydrogel (ALMA 2%), which showed eccentricity values below 0.75 (Fig. 6C). Similarly, the cell area was significantly larger on the stiffest hydrogel ($\sim 197 \mu\text{m}^2$) than on the softest substrate ($\sim 120 \mu\text{m}^2$), suggesting decreased cell interaction with the softer materials (Fig. 6D).

Interestingly, cells located near the edge of the disk-shaped hydrogels consistently exhibited much greater spreading compared to those resided on more central regions (Fig. 6E). This phenomenon was observed for all the three ALMA concentrations. The spreading appeared to be unidirectional towards the edge, evidenced by the cell lamellipodia distribution. This unexpected edge-related cell morphology might be due to a higher local stiffness near the perimeter, potentially caused by increased water evaporation near the edge during hydrogel preparation. However, the local stiffness and composition will need further characterisation in future studies to elucidate the underlying mechanisms.

To further determine the phenotype of macrophages on different ALMA hydrogels, pro- (TNF- α , IL-6) and anti- (IL-10, CCL18) inflammatory cytokines were quantified by using sandwich ELISA (Fig. 7). TNF- α secreted from macrophages cultured on ALMA 6% was significantly higher compared to cells on ALMA 2% and 4%, suggesting a shift towards a pro-inflammatory phenotype on the stiffer substrate. Although no significant difference was observed for IL-6, a trend of increasing secretion with increasing stiffness was observed. In terms of anti-inflammatory cytokines, IL-10 secretion was significantly higher from macrophages cultured on the softest ALMA 2%.

Although no significant difference was observed for CCL18 release, a decreasing trend with increasing stiffness was observed. Overall, the cytokine profile supported the observation that stiffer hydrogels induced a pro-inflammatory phenotype, whereas softer substrates showed an anti-inflammatory activation status.

It is known that high endotoxin levels can induce pro-inflammatory polarisation in macrophages.⁵³ To exclude the possibility that changes in cell behaviour were a consequence of high endotoxin levels, ALMA 6% was prepared and incubated in PBS, simulating the cell culture conditions. After 6 days, endotoxins were quantified and data showed that their level was below the accepted limit of 0.1 EU per mL for *in vitro* immunogenicity assays, which excluded the possibility of the influence of endotoxins on macrophage activation status (Fig. S3).

Discussion

The conflicting reports on macrophage responses to substrate stiffness, along with the relatively high stiffness ranges commonly investigated in the literature, prompted us to study human macrophages on soft hydrogels. We prepared hydrogels with different mechanical properties by varying ALMA concentrations from 2% to 6% w/v. Our results showed that even a modest increase (in absolute value) from ~ 0.25 kPa to ~ 4.5 kPa led to significant changes in human monocyte-derived macrophages. Our results demonstrated that cells cultured on the stiffest substrate (ALMA 6%, ~ 4.5 kPa) showed a more elongated morphology as early as day 3. This feature was also



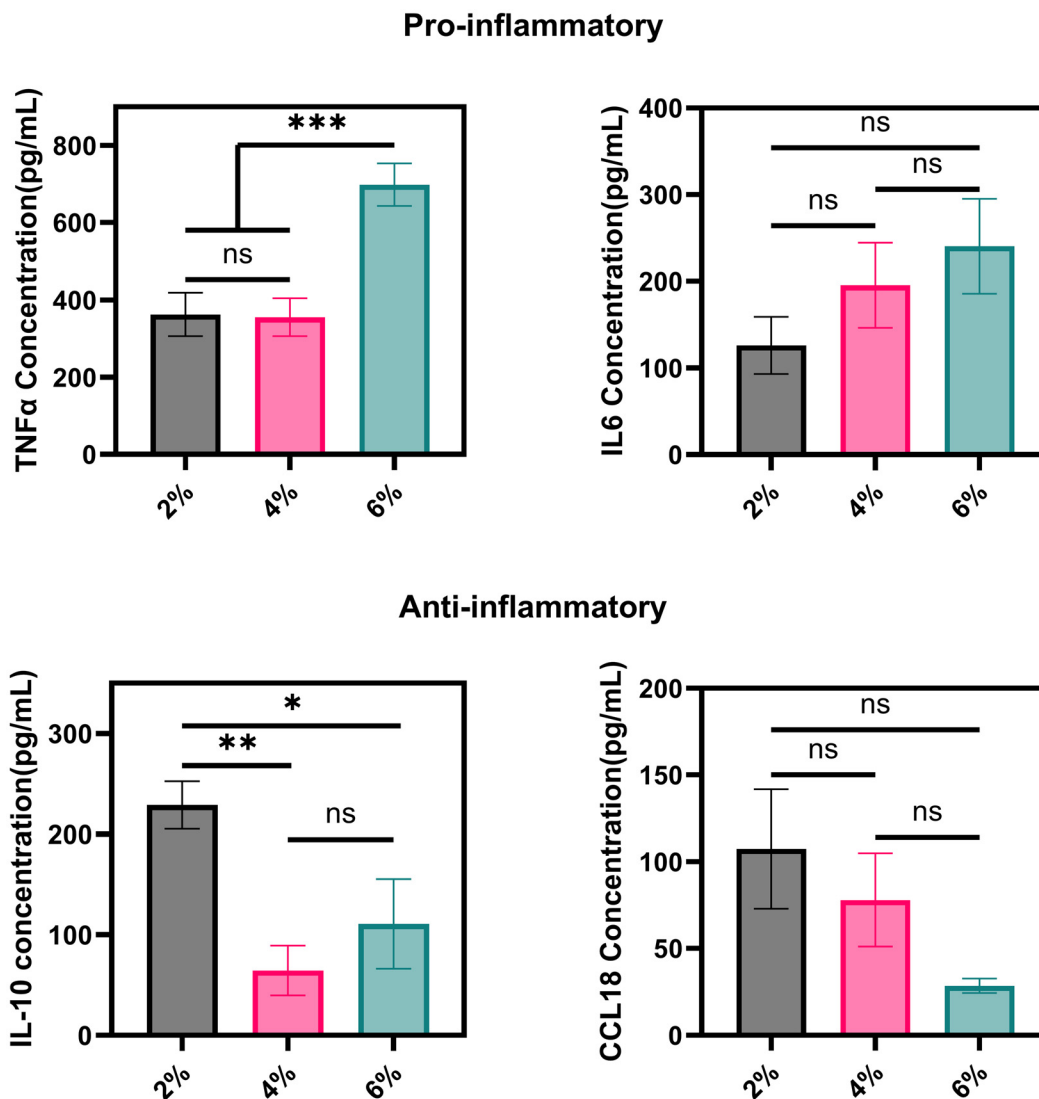


Fig. 7 Quantification of pro- (TNF- α , IL-6) and anti- (IL-10, CCL18) inflammatory cytokines secreted by macrophages at day 6.

associated with a higher calprotectin-to-mannose receptor expression ratio, and a significant increase in pro-inflammatory cytokine release, particularly TNF- α . A stiffness-dependent increase in IL-6 was also observed when moving from soft (ALMA 2%) to stiff (ALMA 6%) hydrogels.

The stiffness-dependent trend observed in our study aligns with several previous reports in which increased stiffness promoted a shift towards M1-like macrophage phenotype. Blakney *et al.* reported increased TNF- α , IL-1 β , and IL-6 expression in macrophages cultured on stiff PEG gels (840 kPa),¹³ while Sridharan *et al.* found greater cell spreading and upregulation of two pro-inflammatory chemokines, such as CXCL11 and CCL20, on stiff polyacrylamide gels (323 kPa).¹⁴ In both studies, softer gels (>11 kPa) promoted an anti-inflammatory phenotype. Similarly, Zhuang *et al.* showed that increasing GelMA stiffness (2–29 kPa) enhanced inflammatory signalling and M1 polarisation.¹⁵ Our findings are also consistent with

those of Previtera *et al.*, who showed reduced pro-inflammatory mediator release on soft gels (0.3 kPa) compared to stiff ones (230 kPa).¹⁶ Although these reported findings align with our data, it is important to note that most of the previously defined “soft” substrates are still 2–3 orders of magnitude stiffer than our ALMA 2% hydrogel (~0.25 kPa), with the exception of the soft gel used by Previtera *et al.* These reported soft hydrogels are even at least 2-fold stiffer than our stiffest gel (ALMA 6%, ~4.5 kPa). Notably, our findings extend and expand the existing knowledge by demonstrating that stiffness-driven M1 activation is not limited to high-stiffness substrates (>10 kPa) but can also arise within the low-kilopascal range. This is particularly relevant in the context of soft tissues, both in healthy and fibrotic conditions. For example, in the liver and brain, stiffness can increase from 0.3–1 kPa under healthy conditions to >25 kPa during fibrosis.^{54,55} These results highlight the need for comparative studies which focus on the low-



kPa range to elucidate how subtle changes in the mechanical properties of soft tissues influence macrophage behaviour.

Several studies reported an opposite trend to what we and others observed, showing a pro-inflammatory phenotype associated with softer gels.^{18–22} Such discrepancies might depend on other factors than stiffness alone. In fact, stiffness is not the only mechanical property of hydrogels. Viscoelasticity is another key feature⁵⁶ and changing the degree of crosslinking in a 3D polymer network can alter the viscoelastic properties of hydrogels.^{57–59} Viscoelasticity has been shown to modulate mechano-sensitive molecular pathways in various cell types,⁵¹ such as fibroblasts and cancer cells,⁵⁹ hepatocytes,⁶⁰ and human induced pluripotent stem cells.⁶¹ Kalashnikov N. *et al.* have directly addressed how viscoelastic cues modulate THP-1 macrophage morphology and functions, revealing that more viscous substrates induce a rounder morphology and a reduced phagocytic activity.⁴⁹ Despite this, studies examining the role of viscoelasticity in macrophage behaviour remain scarce. Further research is needed to elucidate how viscoelastic properties affect macrophages, particularly in the context of FBR.

Another factor that may explain the divergent results is the source of macrophages used in different studies.⁶² It is worth noting that most studies used murine bone marrow-derived macrophages, RAW264.7 cells¹⁸ or NR8383 rat alveolar macrophages,¹⁹ all of which may exhibit different sensitivities and responses to mechanical cues compared to human macrophages. Only a few studies have used primary monocyte-derived macrophages, and interestingly they showed trends opposite to ours. Guenther *et al.* showed that increased stiffness led to a M2-like phenotype, with macrophages on stiffer substrates expressing higher levels of CD206 and MMP13.²³ However, their study involved coculture with cancer cells, introducing additional variables such as tumour-macrophage crosstalk that can independently affect macrophage phenotype. Furthermore, the use of a 3D culture system in their study adds complexity, as dimensionality is also known to impact macrophage behaviour.⁶³ Similarly, Scott *et al.* reported that human macrophages cultured on stiffer RGD-modified-PEG-based gels (10.3 kPa) displayed a shift towards an anti-inflammatory phenotype.²⁴ Adlerz *et al.* also studied human monocyte-derived macrophages on fibronectin-coated polyacrylamide gels of varying stiffness.²⁵ While their findings addressed morphological changes, they did not include key phenotypic indicators such as surface marker expression or cytokine secretion, making it difficult to determine whether substrate stiffness promoted a pro- or anti-inflammatory state.

Surface chemistry plays a critical role in modulating cell-biomaterial interactions, primarily by influencing the adsorption of proteins from serum or culture media onto the substrate surface.⁶⁴ The amount, composition, and conformation of the adsorbed protein layer vary depending on the material's chemical properties, and this layer critically determines the presentation of adhesive motifs to cells. Hydrogels can also be functionalized with peptides, such as RGD-containing sequences,²⁴ or extracellular matrix proteins, such as fibronec-

tin or collagen,^{21,25} that favour cell attachment, promoting a stronger integrin engagement compared to unmodified hydrogels, which rely solely on non-specifically adsorbed proteins. These variations directly affect how integrins engage with the substrate, as integrin-based adhesion complexes serve as key mechano-transduction bridges between the extracellular environment and the cytoskeleton.⁶⁵ As a result, variations in protein adsorption and differences in surface densities of binding motifs can influence cell adhesion and mechano-transduction pathways, independently from the mechanical properties of the gels. Ultimately, these changes can affect gene expression and functional responses in cells, including macrophages, as demonstrated in previous studies.^{66–69} This may partly explain the divergent polarisation outcomes observed across different studies using hydrogels with comparable stiffness but different surface chemistries.

Other features, such as surface roughness and mesh size, can influence macrophage responses, as previously reported in the literature.^{70,71} However, our data indicate that their contribution is minimal in our system, with stiffness emerging as the dominant factor shaping cellular behaviour. For example, although ALMA 4% and 6% exhibited comparable roughness but different stiffnesses, macrophages cultured on ALMA 6% displayed significantly higher calprotectin/MR ratio, greater spreading, and increased TNF- α release. These effects can therefore be attributed primarily to stiffness, as topographical differences were negligible. Conversely, while ALMA 2% and 4% hydrogels differed significantly in both roughness and mesh sizes, their storage moduli (G') were not significantly different, and macrophage responses – including calprotectin/MR ratio, eccentricity, cell spreading, and TNF- α , IL-6, and CCL18 release – remained largely similar. Taken together, these findings suggest that nanoscale variations in roughness and mesh size exert little influence on macrophage behaviour in this system, whereas stiffness consistently acts as the key mechanical determinant of macrophage phenotype.

Conclusions

In this study, we show that the stiffness of ALMA hydrogels significantly influences the behaviour of human primary macrophages. Specifically, stiffer hydrogels (~4.5 kPa) promoted a pro-inflammatory phenotype. This was evidenced by the elongated morphology observed on the stiffest hydrogel (ALMA 6%) and a higher calprotectin-to-mannose receptor ratio, indicating a stiffness-dependent, mechano-sensitive shift in macrophage behaviour. These findings were further supported by cytokine profiling, which showed an elevated secretion of TNF- α and IL-6 on stiffer hydrogels compared to softer substrates (~258 Pa). Importantly, this pro-inflammatory response was observed even within the range of soft tissue-relevant stiffness, highlighting the sensitivity of macrophages to subtle mechanical variations. Future studies should explore additional material properties such as viscoelasticity, surface chemistry, and protein adsorption to further elucidate the



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