

Article

The Collapse of the Collagen Sponge Microstructure Triggers an Inflammatory Response of Macrophages via the *Itgαvβ3/5-Src-RhoC-NF-κB* Axis

Zefeng Guo ^{1,2,3}, Mengxi Su ^{1,2,3}, Meihua Mai ^{1,2,3}, Tianze Lin ^{1,2,3}, Xinyi Yang ^{1,2,3}, Shiyu Wu ^{1,2,3,*} and Zhuofan Chen ^{1,2,3,*}

¹ Hospital of Stomatology, Sun Yat-Sen University, Guangzhou 510055, China

² Guanghua School of Stomatology, Sun Yat-Sen University, Guangzhou 510055, China

³ Guangdong Provincial Key Laboratory of Stomatology, Guangzhou 510055, China

* Correspondence: wushy55@mail.sysu.edu.cn (S.W.); chzhuof@mail.sysu.edu.cn (Z.C.)

Abstract

Collagen sponges are widely used for oral tissue regeneration, due to their extracellular matrix-mimetic architecture and excellent biocompatibility. However, in practical biomedical applications, collagen sponges may exhibit hydration-induced structural instability, and there can be associated inflammatory responses under physiological conditions, potentially compromising their regenerative performance. In this study, we investigated how two cross-linking strategies—transglutaminase (TG) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide/N-hydroxysuccinimide (EDC/NHS)—modulate the structural stability and inflammatory profiles of collagen sponges. TG-cross-linked sponges exhibited microstructural collapse, associated with macrophage activation and engagement of the *Itgαvβ3/5-Src-RhoC-NF-κB* signaling axis. In contrast, EDC/NHS-cross-linked sponges preserved a stable porous architecture, effectively suppressing this signaling cascade and establishing a low-inflammatory microenvironment. These findings elucidate a key mechanism by which cross-linking regulates the microstructural integrity of collagen scaffolds and provides in vitro-derived preliminary design principles for developing next-generation collagen biomaterials with low-inflammatory properties.

Keywords: collagen sponge; cross-linking; integrin; macrophage; inflammatory response

1. Introduction

Collagen-based products are widely used in oral tissue regeneration. In terms of soft tissue regeneration, gelatin sponges can be used for hemostasis [1,2], while collagen sponge-like materials can promote the healing of soft tissue wounds [3,4]. In the field of bone tissue regeneration, they serve as a barrier membrane [5,6] to prevent soft tissue from growing. Collagen can also be mixed with bone substitutes to form bone–collagen composites [7,8], thereby regulating the process of bone regeneration alongside the bone graft material.

Immunological research has confirmed that the initial immune response elicited by biomaterials is crucial for tissue regeneration. An appropriate immune response may recruit regenerative cells [9], whereas an excessive inflammatory reaction can impede the regenerative process [10]. A previous study has shown that collagen sponges may induce significant inflammatory reactions [11,12]. In vitro studies on collagen membranes have also confirmed that macrophages exhibit significant upregulation of inflammatory genes in direct



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in vitro culture models [13]. Therefore, it is important to clarify the factors that influence the immunomodulatory effects of collagen sponges and the underlying mechanisms.

The malleability of collagen materials is a crucial property for clinical applications. Increased malleability enables collagen–bone composites to exhibit improved handling characteristics. It also allows collagen sponges to be effectively compressed into soft tissue wounds, which facilitates hemostasis. This property is closely related to the collagen's microstructure. Recent studies have demonstrated that the microarchitecture of collagen can directly modulate the cellular immune responses [14,15]. However, the precise mechanism by which the mechanical properties of collagen sponge materials regulate immunity remains incompletely understood.

This study aims to investigate the immunomodulatory effects and underlying mechanisms of a commercially available transglutaminase (TG)-cross-linked collagen sponge on macrophages (collagen sponge (TG)). In addition, an EDC/NHS-cross-linked collagen sponge (collagen sponge (E/N)) was fabricated to assess how the microstructure influences the immune response. These findings will clarify the immunomodulatory properties and mechanisms of collagen sponges in vitro, providing a foundation for developing novel collagen-based biomaterials with controllable inflammatory responses.

2. Materials and Methods

2.1. Preparation of Collagen Sponges

All reagents used in the study are listed in Supplementary Table S1. A commercially available cross-linked collagen sponge (model: BD-CS-01; Wuxi Beidi Bioengineering Co., Ltd., Wuxi, China) was used as the control material and is hereafter referred to as a collagen sponge (TG). According to the manufacturer's specifications, the sponge is a medical-grade collagen material derived from porcine skin and purified by acid extraction followed by enzymatic digestion to remove non-collagenous components. Prior to freeze-drying, collagen molecules were enzymatically cross-linked using transglutaminase (TG), resulting in the formation of covalent ϵ -(γ -glutamyl) lysine bonds between adjacent collagen fibers. The collagen sponges used in this study had standardized dimensions of 50 mm $\times$ 50 mm $\times$ 2 mm (length $\times$ width $\times$ thickness). The collagen sponges (TG) were supplied by the manufacturer as a pre-sterilized product. The material was used directly in all experiments without additional sterilization.

Collagen sponges (E/N) were fabricated using pigskin raw material purchased from (Daqing Yikouzhu Group Co., Ltd., Zhaozhou, China), a certified enterprise compliant with national slaughter and food safety regulations. The pigskin was obtained through standardized commercial slaughter processes in accordance with China's Regulations on the Administration of Pig Slaughter and Hygienic Specifications for Pig Slaughter Processing. The raw material, porcine skin, was initially defatted by cleaning with ultrapure water (18.2 M Ω ·cm at 25 °C) via an on-site laboratory water purification system (Millipore Milli-Q system), trimming to size, and immersing in a sodium hydroxide solution to saponify residual lipids, followed by thorough rinsing. To remove salt-soluble proteins, the defatted skin was soaked in a sodium chloride solution and then washed with pure water. Collagen extraction was performed using acid dissolution with acetic acid. The pH was adjusted to 7.0 before tissue homogenization. The homogenization process was performed using a Joyoung Y10 Fully Automatic Self-Cleaning High-Speed Homogenizer (Joyoung Co., Ltd., Hangzhou, China). The homogenized mixture underwent pepsin digestion, gradient filtration, pH adjustment, and salting out to isolate the collagen. The resulting collagen precipitate was redissolved in acetic acid and purified by dialysis at room temperature. To fabricate the collagen material, the prepared collagen solution was mixed with 10% gelatin. After initial shaping via gradient freezing and lyophilization, the blocks were

chemically cross-linked using an EDC/NHS system. A cross-linking solution was prepared in 75% ethanol containing 11.5 mg/mL EDC and 2.76 mg/mL NHS. A volume of 1 mL of this solution was added per 10 mg of collagen and incubated for 2 h. The reaction was quenched by adding 0.1 mol/L disodium hydrogen phosphate dodecahydrate solution, and the mixture was allowed to stand for 30 min. Samples were pre-frozen in a gradient from $-20\text{ }^{\circ}\text{C}$ to $-80\text{ }^{\circ}\text{C}$ for 20 h. The pre-frozen samples were then transferred to an (Alpha 2-4 LD plus vacuum freeze dryer Osterode am Harz, Germany). The vacuum was gradually reduced from 500 mbar to 0.751 mbar. The shelf temperature was ramped from $-80\text{ }^{\circ}\text{C}$ to $0\text{ }^{\circ}\text{C}$ at $0.5\text{ }^{\circ}\text{C}/\text{min}$ (held for 10 h), then increased to $25\text{ }^{\circ}\text{C}$ at $1\text{ }^{\circ}\text{C}/\text{min}$ for 4.8 h, completing the 20 h freeze-drying process.

All collagen sponges used for cell experiments were sterilized prior to use. The self-prepared EDC/NHS-cross-linked collagen scaffolds were sterilized by γ -ray irradiation before cell culture. Sterilization was performed by Guangzhou Huada Biotechnology Co., Ltd. (Guangzhou, China) using a cobalt-60 (^{60}Co) γ -ray source, with the total absorbed dose range of 25–27 kGy for medical device sterilization.

2.2. Physicochemical Examination of the Collagen Sponges

Cell adhesion and morphology on collagen sponges were observed using a light microscope (OLYMPUS CX23, Tokyo, Japan) after cell culture. For sample preparation, collagen sponges were sectioned into 24-well plate-compatible sizes using a sterile punch; logarithmic-phase RAW264.7 cells were centrifuged (1000 rpm, 5 min), resuspended in complete medium to prepare a single-cell suspension (density see subsequent cell culture method), and an appropriate volume was seeded onto collagen sponges. Fresh complete medium was added to reach 1 mL per well, followed by incubation at $37\text{ }^{\circ}\text{C}$ with 5% CO_2 for 24 h, and observation was conducted at $40\times$ magnification ($\text{NA} = 0.65$) after incubation.

For scanning electron microscopy (Hitachi SU8010, Hangzhou, China) observation of macrophage morphology, cells cultured on collagen scaffolds were fixed with 2.5% glutaraldehyde for 4 h at room temperature, followed by thorough rinsing with 0.1 M phosphate buffer (pH 7.4). Samples were then dehydrated through a graded ethanol series (30%, 50%, 70%, and 90%, 15 min each), followed by three changes of absolute ethanol. Ethanol was subsequently replaced with tert-butanol through stepwise immersion, and samples were pre-frozen at $-20\text{ }^{\circ}\text{C}$. Freeze-drying was performed using a vacuum freeze dryer (Alpha 2–4 LD plus, Osterode am Harz, Germany) under programmed vacuum and temperature conditions. After drying, samples were equilibrated to room temperature under vacuum prior to SEM observation. The morphology of the collagen materials, both before and after cell culture, was examined using a scanning electron microscope (SEM; TESCAN MIRA LMS, Brno, Czech Republic). Collagen and collagen sponge samples were cut into rectangular specimens ($4\text{ mm} \times 2\text{ mm}$). The samples were mounted on SEM stubs using conductive adhesive tape. The samples were then sputter-coated with platinum ions and observed at fixed magnifications.

For elemental composition analysis, an energy-dispersive X-ray spectroscopy (EDS) system (Quantax75, Hitachi High-Tech, Hangzhou, China) coupled with SEM was used at an accelerating voltage of 15 kV. Representative areas on the sample surface were selected for elemental mapping to assess the distribution and relative content of major elements, evaluating the effect of the cross-linking process on the elemental composition.

Microstructural collapse is defined as the deterioration of the collagen sponges' porous architecture under physiological-mimicking conditions. Quantitative analysis of scaffold microstructural parameters was performed using scanning electron microscopy (SEM) images analyzed with ImageJ software (version 1.53t, National Institutes of Health, Bethesda, MD, USA). SEM images were acquired at a fixed magnification to ensure consistency. For

each independent scaffold sample, five non-overlapping fields of view were randomly selected from different regions of the scaffold. Three independent scaffold samples were analyzed per group ($n = 3$), yielding at least 15 fields of view per group. For porosity analysis, SEM images were converted to 8-bit grayscale and binarized using a consistent global threshold in ImageJ. Identical threshold parameters were applied to all images within the same experimental batch. Porosity was calculated as the percentage of pore area relative to the total image area using the “Analyze Particles” function. For scaffold shrinkage analysis, three independent scaffold samples per group were imaged before and after cell culture. The base area of each scaffold was measured using calibrated SEM images in ImageJ, and the shrinkage rate was calculated as follows: Shrinkage rate (%) = [(initial base area – post-treatment base area)/initial base area] $\times$ 100%.

The mechanical properties of the collagen samples were assessed using a universal testing machine. Rectangular samples (5 mm $\times$ 6 mm) were prepared and immersed in DMEM for 24 h to simulate the cellular environment. The elastic modulus was measured under compression at a rate of 0.5 mm/min using three biological replicates per group. The stress–strain curve was generated using TRAPEZIUM LITE X software (version 1.5.0c).

The structural characteristics of collagen and collagen sponge samples were analyzed using a Fourier-transform attenuated total reflectance infrared (ATR-FTIR) spectrometer. Spectra were acquired over the range of 4000–400 cm^{-1} with a resolution of 4 cm^{-1} , using 32 scans per sample. The obtained spectra were processed using OriginPro 2024 software. Linear baseline correction was applied across the full spectral range to eliminate background drift, followed by vector normalization based on the Amide I band (1620–1680 cm^{-1}) to minimize intensity variations arising from differences in sample thickness or contact conditions. The processed spectra were used to identify characteristic collagen peaks and for comparative analysis between TG and E/N-cross-linked collagen sponges.

2.3. Cell Culture and Cell–Material Co-Incubation

RAW264.7 murine macrophages (CL-0190; Procell, Wuhan, China) were cultured in Dulbecco’s Modified Eagle Medium (DMEM; C11885500BT, Thermo Fisher Scientific, Shanghai, China) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS; FSP500, ExCell Bio, Montevideo, Uruguay) and 1% (v/v) penicillin–streptomycin (15,140,122, Thermo Fisher Scientific, Waltham, MA, USA). FBS heat inactivation was carried out at 56 °C for 30 min. Cells were maintained at 37 °C in a humidified 5% CO_2 incubator. When cultures reached 80–90% confluence, passaging was performed at a 1:3 ratio. For co-incubation experiments, 1×10^4 cells were seeded per well in 24-well plates and incubated on collagen sponges for 24 h.

2.4. Cytotoxicity Assay

The cytocompatibility of collagen sponges was assessed via a standardized in vitro evaluation system. The cytotoxicity assay was conducted using the cell counting kit-8 (CCK-8) (Catalog No. CK04, Toyobo Co., Ltd., Osaka, Japan). Before cell seeding, collagen sponges were sectioned into 5 mm $\times$ 5 mm fragments using a sterile punch to match the well size of a 96-well plate. Subsequently, macrophages were resuspended and seeded into the 96-well plate at a density of 2000 cells per well. On day 1 and day 3, the culture medium was replaced with DMEM solution supplemented with 10% (v/v) CCK-8, and the cells were incubated for 1 h. The absorbance (OD) of each well was determined at a wavelength of 450 nm using a microplate reader (Epoch2, Winooski, VT, USA). Each group was analyzed in quintuplicate.

2.5. Integrin Inhibitor Pretreatment

RAW264.7 cells were seeded at 10,000 cells per well in 24-well plates and allowed to adhere for 8–12 h at 37 °C. The integrin $\alpha v\beta 3/\alpha v\beta 5$ inhibitor Cilengitide (EMD 121974) was prepared in UltraPure™ DNase/RNase-Free Distilled Water (10977015, Invitrogen, Thermo Fisher, Waltham, MA, USA) and diluted in complete medium to a final concentration of 10 $\mu\text{g}/\text{mL}$. For integrin inhibition experiments, a handling-matched control design was applied. Cells were seeded at the same density into 12 wells of a 24-well plate. After 8 h of adhesion, Cilengitide was added to 3 wells for a 12 h pretreatment, while the remaining wells were incubated without the inhibitor under identical conditions. Following pretreatment, the medium was refreshed, and cells were incubated for an additional 12 h with or without Cilengitide according to group assignment. Cells from all wells were then gently detached using the same procedure and re-seeded onto collagen sponges for a 24 h co-incubation period.

2.6. RNA Extraction and RT-qPCR

Total RNA was isolated using the RNA-Quick Purification Kit (RN001, ESscience, Shanghai, China). RNA concentration and purity were evaluated by measuring absorbance at 260 nm and 280 nm. A total of 500 ng RNA was reverse-transcribed using the Synthesis SuperMix Kit (11141ES60, YEASEN, Shanghai, China). Primers were designed based on NCBI cDNA sequences and verified using (BLAST, 2.14.1). RT-qPCR was performed on a QuantStudio 7 Real-Time PCR System (Applied Biosystems, Waltham, MA, USA) using SYBR Green Master Mix (11202ES08, YEASEN, Shanghai, China). GAPDH served as the internal control. All samples were analyzed in triplicate, and melt-curve analysis was performed.

2.7. RNA Sequencing and Bioinformatics Analysis

After 24 h of co-incubation, macrophages were harvested, and total RNA was extracted using TRIzol reagent according to the manufacturer's instructions. Transcriptomic analysis included two experimental groups (blank control and treated collagen sponges), with three independent biological replicates per group ($n = 3$), yielding six RNA-seq samples. Library preparation and sequencing were performed by BGI Genomics. Briefly, mRNA was enriched using oligo (dT) magnetic beads, fragmented, and reverse-transcribed with random N6 primers to generate double-stranded cDNA. Following end repair, 3' adenylation, adapter ligation, and PCR amplification, single-stranded circular DNA libraries were constructed to generate DNA nanoballs (DNBs). Paired-end sequencing (PE150) was conducted on the DNBSEQ platform, with an average sequencing depth of approximately 6.6 Gb per sample ($Q20 \geq 98.6\%$, $Q30 \geq 92.9\%$).

Raw sequencing reads were filtered for quality using SOAPnuke (v1.5.6) and aligned to the mouse reference genome (GRCm39) using HISAT2 (v2.2.1). Gene expression levels were quantified as transcripts per million (TPM) using RSEM (v1.2.28). Differential gene expression analysis between groups was performed using the DESeq2 R package (v1.40.2), with thresholds of $|\log_2 \text{fold change}| > 1.0$ and adjusted p value < 0.05 .

Global transcriptional patterns were visualized by principal component analysis (PCA), volcano plots, and hierarchical clustering heatmaps using the Dr.Tom bioinformatics analysis platform (BGI Group; <https://biosys.bgi.com>, accessed on 17 February 2024).

To investigate cytoskeleton-associated transcriptional changes, cytoskeleton-related genes were first identified based on Gene Ontology (GO) Cellular Component annotations associated with cytoskeletal structures, including the actin cytoskeleton, microtubule cytoskeleton, centrosome, and spindle. These genes were then intersected with the differentially expressed genes to generate a cytoskeleton-related DEG subset.

Functional enrichment analyses were performed using the ClusterProfiler R package (v4.6.2). GO enrichment analysis referenced the Mouse GO Database (Release 202401), covering Biological Process, Cellular Component, and Molecular Function, while KEGG pathway analysis used the KEGG PATHWAY Database (Release 108.0, *Mus musculus*, 'mmu'). Enrichment results were considered significant at an adjusted p value < 0.05 and gene count ≥ 3 (Benjamini–Hochberg correction). GO functional classification plots and KEGG pathway bubble plots were generated using TBtools software (v1.100).

Protein–protein interaction (PPI) networks were constructed using the STRING database (v12.0) with a minimum confidence score of 0.7. Only interactions supported by experimental evidence or curated database annotations were retained. Network topology analysis and visualization were performed using Cytoscape software (v3.9.1).

2.8. ELISA Detection

Supernatants collected at 24 h and 48 h of co-incubation were analyzed using commercial ELISA kits for inflammatory cytokines (TNF- α kit: CSB-E04741m-IS, IL-6 kit: CSB-E04640r, Wuhan, China). Reagents and samples were equilibrated to room temperature for 30 min. After preparation of standards and sample dilutions, 100 μ L of each was added into wells and incubated for 2 h at 37 °C. After washing, 100 μ L of biotin-conjugated antibody solution was added for 1 h, followed by HRP-avidin incubation for another hour. TMB substrate (90 μ L) was applied for color development for 15–30 min in the dark. After adding stop solution (50 μ L), absorbance was recorded at 450 nm, and concentrations were calculated using the standard curve.

2.9. Statistical Analysis

Statistical analyses were carried out utilizing GraphPad Prism 9.0 software (GraphPad Software Inc., San Diego, CA, USA). For quantitative data, homogeneity of variance was assessed through Levene's test prior to formal comparisons, and all data were presented as the mean $\pm$ standard deviation (SD). When comparing two independent groups, the unpaired two-tailed Student's t -test was employed to determine differences; for comparisons involving three groups, one-way analysis of variance (one-way ANOVA) was performed, with subsequent Tukey's post hoc test used to identify specific inter-group variations. p -value less than 0.05 was defined as statistically significant, with additional significance levels denoted as * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

3. Results and Discussion

3.1. Microstructural Collapse of Collagen Sponges Induces a Pro-Inflammatory Response in Macrophages

Collagen sponges tend to undergo compressional deformation upon hydration, facilitating their easy packing into tissue wound sites [16,17]. To elucidate the morphological changes of collagen sponges post wetting, we first characterized the materials at both macroscopic and microscopic scales. Macroscopically, the material volume decreased significantly 24 h after immersion in DMEM medium (Figure 1a). For microscopic analysis, scanning electron microscopy (SEM) samples were prepared, which revealed a marked reduction in the porosity of the sponge (Figure 1b,c). Specifically, the porosity decreased by $76.73\% \pm 2.1\%$ (Supplementary Table S2), indicating the collapse of their microstructure.

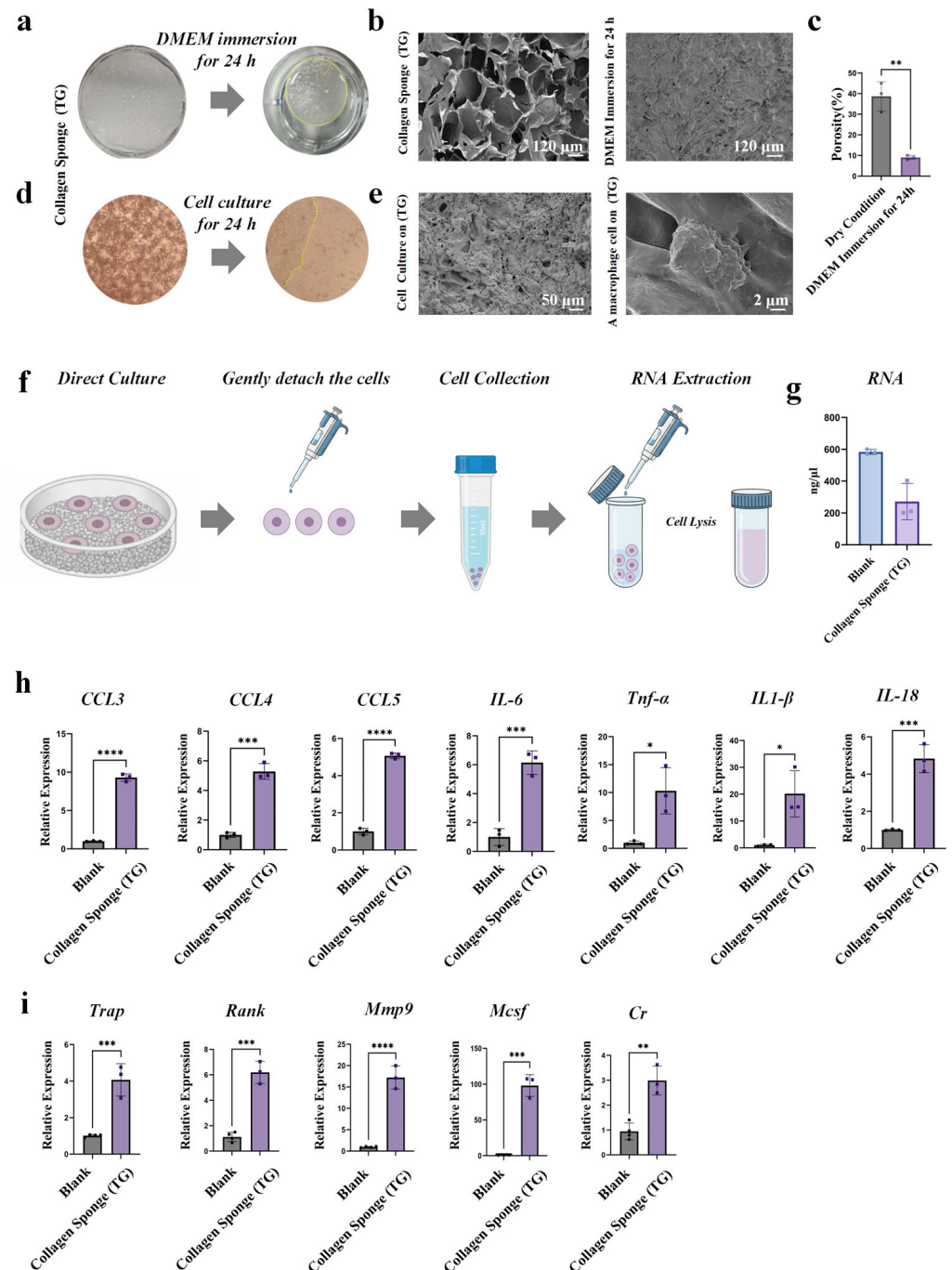


Figure 1. The microstructural collapse of the collagen sponges induced an obvious inflammatory response. (a) Gross view showing significant collagen sponge shrinkage after 24 h of DMEM immersion. The yellow dashed line indicates the area change after 24 h. (b) SEM revealed a significantly reduced porosity in the collagen sponges. (c) Quantification of collagen sponge (TG) porosity under dry conditions and DMEM immersion for 24 h ($n = 3$). (d) Gross view showing significant collagen sponge shrinkage after cell culture. (e) SEM showing compressed and spindle-shaped cell morphology on the collagen sponges. (f) Schematic diagram of extracting RNA from collagen sponges. (g) Sufficient RNA was extracted from cells on collagen sponges ($n = 3$). (h) Quantitative analysis of pro-inflammatory genes in macrophages via RT-qPCR. (i) Quantitative analysis of osteoclastogenic genes in macrophages by RT-qPCR. ($n = 3$). Data are presented as means $\pm$ SD; statistical analysis was performed using the unpaired two-tailed Student's t -test and ANOVA followed by Tukey's multiple comparison post hoc test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

To further investigate the immune response of macrophages cultured on the collapsed collagen sponges, an in vitro cell–material co-incubation model was established. Following cell seeding, the materials exhibited a similar macroscopic contraction (Figure 1d). SEM images showed that macrophages cultured on collapsed collagen sponges exhibited elongated and compressed morphologies, suggesting a potential morphological response of macrophages to the collagen scaffold's mechanical cues [15] and underlying mechanosensing mechanism [18]. (Figure 1e). To obtain sufficient RNA, cells were first gently pipetted and transferred to centrifuge tubes (Figure 1f). Following centrifugation, lysis buffer was added, and high-quality RNA was isolated via standard RNA extraction protocols (Figure 1g). PCR analysis revealed a significant upregulation of macrophage inflammatory factors, including *Ccl3*, *Ccl4*, *Ccl5*, *IL-6*, *TNF- α* , *IL-18*, and *IL-1 β* (Figure 1h). Osteoclast-associated genes (*Trap*, *Rank*, *Mmp-9*, *M-CSF*, and *Cr*) were likewise elevated in the collagen sponge group (Figure 1i). This is consistent with previous in vivo studies demonstrating that collagen sponges induce a significant inflammatory response [11]. Collectively, these experimental results confirm that microstructural collapse of the collagen sponges induces a pro-inflammatory response in macrophages.

3.2. Collagen Sponges Induced Pro-Inflammatory Response in Integrin-Dependent Manner

To further investigate the signaling pathways underlying the activation of macrophage inflammatory responses, a transcriptome analysis was performed. Principal Component Analysis (PCA) showed clear separation between the blank control and collagen sponge groups, indicating that the sponge drives extensive transcriptional alterations (Supplementary Figure S1a). The volcano plot further revealed numerous differentially expressed genes (DEGs), reflecting a coordinated cellular response to the materials (Supplementary Figure S1b). Functional interpretation of these transcriptional shifts was achieved through Gene Ontology (GO) enrichment analysis. GO Biological Process (GO-BP) terms were strongly enriched for inflammatory and immune-related pathways (Figure 2a), which is consistent with the RT-qPCR results. Hierarchical clustering additionally visualized the marked upregulation of representative inflammatory genes in the collagen sponge group (Figure 2b). GO Cellular Component (GO-CC) analysis revealed marked enrichment of integrin complexes, focal adhesions, and plasma membrane-associated compartments (Figure 2c). Given the well-established role of integrins in orchestrating cytoskeletal organization and mechanotransduction [19–21], we further performed GO enrichment using a predefined cytoskeleton-related gene set, which demonstrated significant enrichment of actin cytoskeleton and broader cytoskeletal terms in the collagen sponge group (Figure 2d). Consistently, heatmap clustering showed coordinated upregulation of multiple integrin family genes (Figure 2e). Moreover, the Protein–Protein Interaction (PPI) network further illustrates the interconnected architecture linking integrins, the actin cytoskeleton, focal adhesion-associated proteins, and inflammation-related regulators (Figure 2f). These findings collectively support the concept that integrins may function as primary mechanosensors that convert physical cues derived from the collagen sponge collapse into downstream biochemical signaling, which is consistent with a previous study [22].

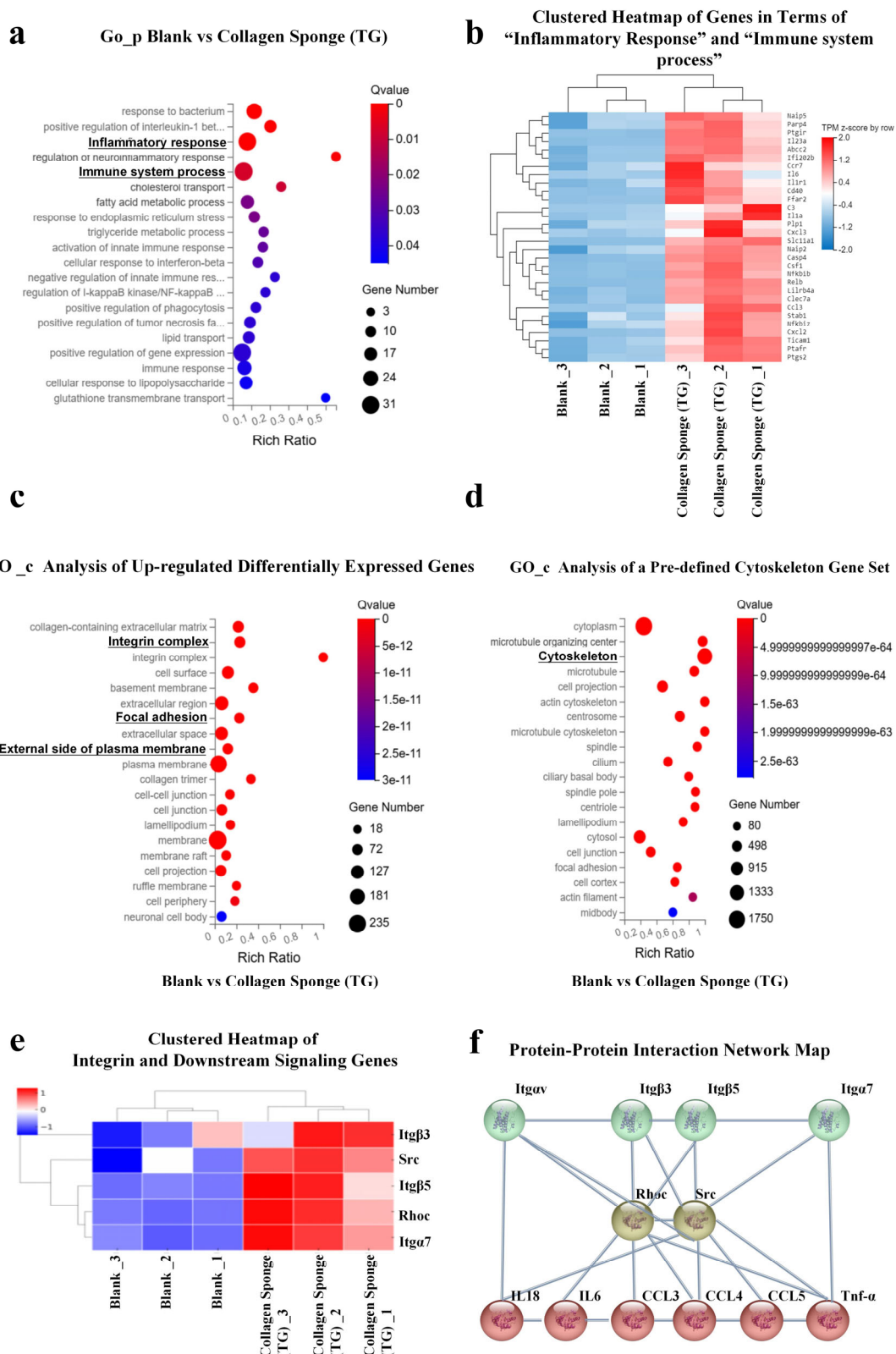


Figure 2. Transcriptomic analysis of macrophages cultured on collagen sponges ($n = 3$). (a) GO_BP enrichment of collagen sponge (TG) vs. blank group. (b) Heatmap analysis showed significant differential expression of inflammatory cytokines. (c) GO_c Analysis of Upregulated Differentially Expressed Genes. (d) GO_c Analysis of a Predefined Cytoskeleton Gene Set. (e) Clustered Heatmap of Integrin and Downstream Signaling Genes. (f) Protein–Protein Interaction Network Map. Differentially expressed RNAs with $|\log_2(FC)|$ value > 1 and adjusted p -value < 0.05 were considered significant for analysis.

As transcriptomic profiling revealed the coordinated upregulation of multiple integrin genes together with *Src* and the cytoskeletal regulator *RhoC* in macrophages, we propose the hypothesis that collagen sponge-induced inflammatory responses are associated with activation of the *Itgav* β 3/5-*Src*-*RhoC*-*NF- κ B* axis. Therefore, an integrin inhibition model was established using the *Itgav* β 3/5 inhibitor Cilengitide (Figure 3a). As a result, the inhibition pretreatment effectively suppressed the integrin gene expression, along with the *Src* and *RhoC* genes (Figure 3b). Substantial downregulation of key pro-inflammatory markers, including *TNF- α* , *IL-1 α* , *IL-6*, *IL-1 β* , and *Nf- κ B*, was also detected (Figure 3c). Consistent with these transcriptional changes, ELISA revealed a significant reduction in secreted *TNF- α* protein levels (Figure 3d). Together, these findings demonstrate that integrin activity is required for the pro-inflammatory program induced by the collapsed collagen sponges. The structural collapse of the collagen sponges may generate physical alterations in the microenvironment, exerting mechanical forces on adherent macrophages [23–25]. These forces are sensed by integrins α v β 3/ β 5, and this mechanosensing is associated with upregulated expression of *Src*-, *RhoC*-, and *NF- κ B*-related genes, consistent with a pro-inflammatory transcriptional program. This is consistent with previous evidence that integrin α v β 3 directly regulates macrophage inflammatory signaling via PI3K/Akt-dependent *NF- κ B* activation [19].

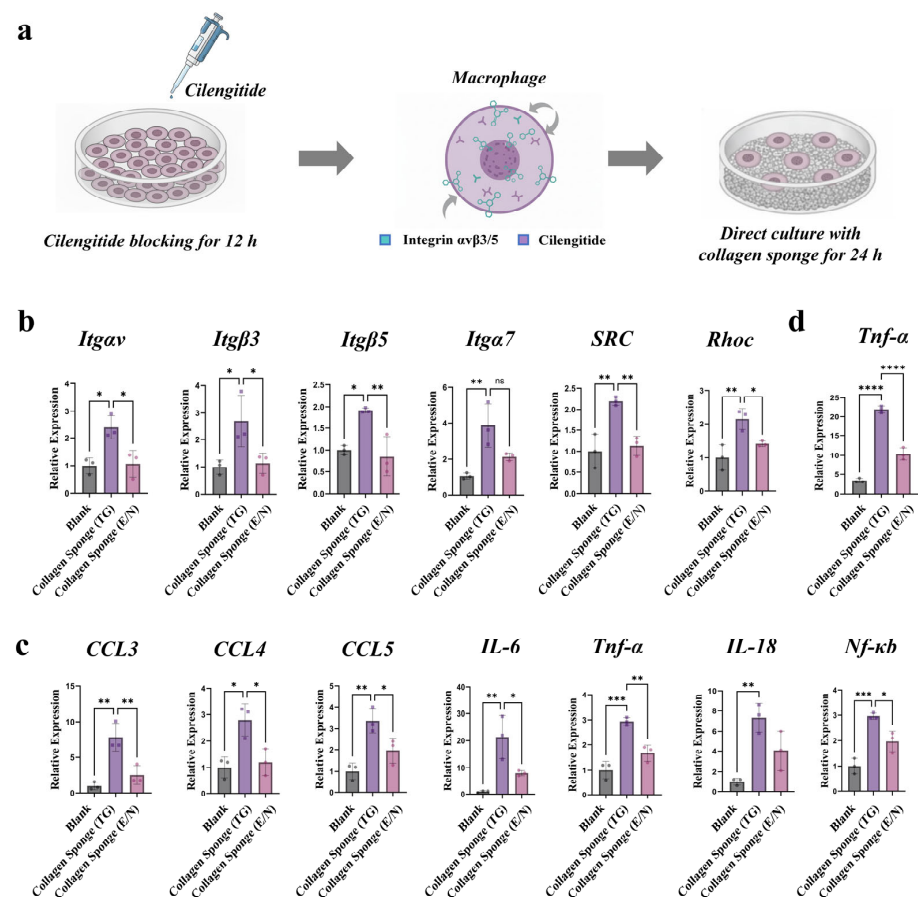


Figure 3. Collagen sponges induced a pro-inflammatory response in an integrin-dependent manner. (a) A schematic diagram of integrin α v β 3/ β 5 blocking assay. (b) Integrin and its downstream genes expression in inhibitor pretreated macrophages by qPCR ($n = 3$). (c) Typical pro-inflammatory and *Nf- κ B* genes expression by qPCR ($n = 3$). (d) ELISA detection of *TNF- α* ($n = 3$). Data are presented as the mean $\pm$ SD; statistical analysis was performed using the unpaired two-tailed Student's *t*-test or ANOVA followed by Tukey's multiple comparison post hoc test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

3.3. Preparation and Characterization of EDC/NHS-Cross-Linked Collagen Sponges

Given the potential impact of microstructural collapse on macrophage inflammatory responses, a collagen sponge with enhanced structural stability was prepared to verify the effect of the microstructure. Appropriate application of cross-linking agents can efficiently enhance the stability of biomaterials [26]. Thus, EDC/NHS-cross-linked collagen sponges sourced from porcine skin were prepared (Figure 4a) [27–30].

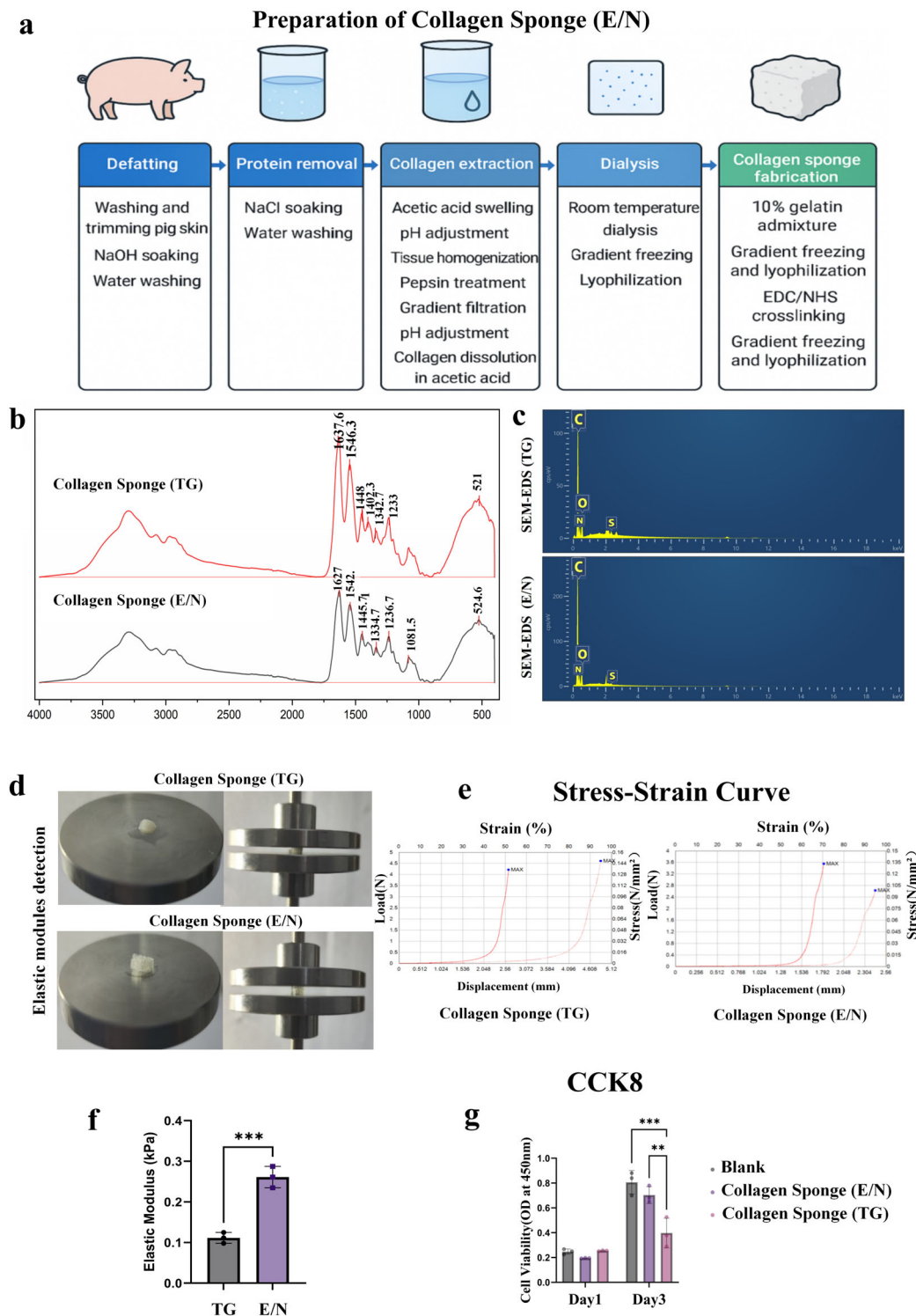


Figure 4. Preparation and characterization of collagen sponge (TG) and (E/N). (a) Preparation of collagen sponges (E/N). (b) FTIR analysis of the collagen sponge. (c) EDS elemental analysis of the

surface of collagen sponge (d) Elastic modulus measurements of the collagen sponge ($n = 3$). (e) Typical stress–strain curves of the collagen sponge (TG) and (E/N) ($n = 3$). (f) Quantification of the elastic modulus of the collagen sponge (TG) and (E/N) ($n = 3$). (g) CCK-8 assay results of the collagen sponge (TG) and (E/N). Data are presented as the mean $\pm$ SD; statistical analysis was performed using the unpaired two-tailed Student's *t*-test or ANOVA followed by Tukey's multiple comparison post hoc test. ** $p < 0.01$, *** $p < 0.001$.

The structural integrity of the synthesized collagen was verified by FTIR (Figure 4b). The characteristic amide peaks—Amide I at 1627 cm^{-1} , Amide II at 1542.1 cm^{-1} , and Amide III in the $1334.7\text{--}1236.7\text{ cm}^{-1}$ —closely match those of the commercial reference sponge. Energy-dispersive X-ray spectroscopy (Figure 4c) further confirmed that both materials are composed predominantly of C, N, O, and S, with no significant contamination, indicating that the cross-linking chemistry did not introduce extraneous elemental impurities. Mechanical testing demonstrated that the EDC/NHS-cross-linked sponge (E/N) exhibited a higher elastic modulus than the commercial collagen sponge (TG), indicating enhanced structural stability (Figure 4d–f, Supplementary Table S2). CCK-8 assays confirmed good cytocompatibility for all tested materials (Figure 4g). Specifically, cell proliferation on the E/N sponge was comparable to the control, whereas proliferation on the TG sponge was significantly reduced, likely due to severe scaffold contraction during culture, which impaired macrophage adhesion and cytoskeletal spreading [15].

3.4. Collagen Sponges with Enhanced Microstructure Stability Downregulated the Inflammatory Response of Macrophages

Building on the elastic modulus measurements, further characterization of the material's macrostructural and microstructural stability was performed. Immersion tests demonstrated that the shrinkage rate of the EDC/NHS-cross-linked collagen sponges (E/N) was significantly lower than that of the commercial collagen sponge (TG) (Figure 5a,b, Supplementary Table S2). Scanning electron microscopy (SEM) observations confirmed that there was no significant difference in the porosity of collagen sponges (E/N) between the pre-hydration and post-hydration states. Collectively, these findings validate that EDC/NHS cross-linking conferred enhanced microstructural stability to collagen sponges relative to the TG group. These structural changes were closely associated with macrophage morphology. Cells cultured on the collagen sponge (TG) displayed an elongated, spindle-like phenotype, whereas macrophages on the collagen sponge (E/N) remained predominantly rounded (Figure 5c). Meanwhile, the porosity of the collagen sponge (E/N) showed no significant reduction (Figure 5d). This pattern is consistent with established evidence that substrate stiffness regulates cytoskeletal organization and cell spreading via integrin-mediated mechanosensing [31], with such mechanical cues often intrinsically linked to matrix microstructure [32]. Together, these findings indicate that microstructural collapse—rather than collagen composition itself—acts as a key determinant shaping macrophage mechanosensing and subsequent inflammatory behavior.

At the molecular level, integrin genes (*Itg α v*/*Itg α 7* and *Itg β 3*/*Itg β 5*) and key regulators of cytoskeletal dynamics (*Src*, *Fak*, and *RhoC*) were markedly upregulated in the collagen sponge (TG) (Figure 5e,f). The expression of these target genes in the collagen sponge (E/N) group was significantly reduced, with no statistically significant difference compared to the blank control group. Consistently, typical inflammatory genes (*NF- κ B*, *IL-6*, *IL-1 β* , *IL-18*, *TNF- α* , *CCL3*, *CCL4*, and *CCL5*) (Figure 5g) and osteoclast-related genes (*Rank*, *Cr*, *Mmp9*, and *Trap*) (Figure 5h) were also significantly downregulated in the collagen sponge (E/N) group. Protein-level expression of TNF- α and IL-6 further supported these findings (Figure 5i).

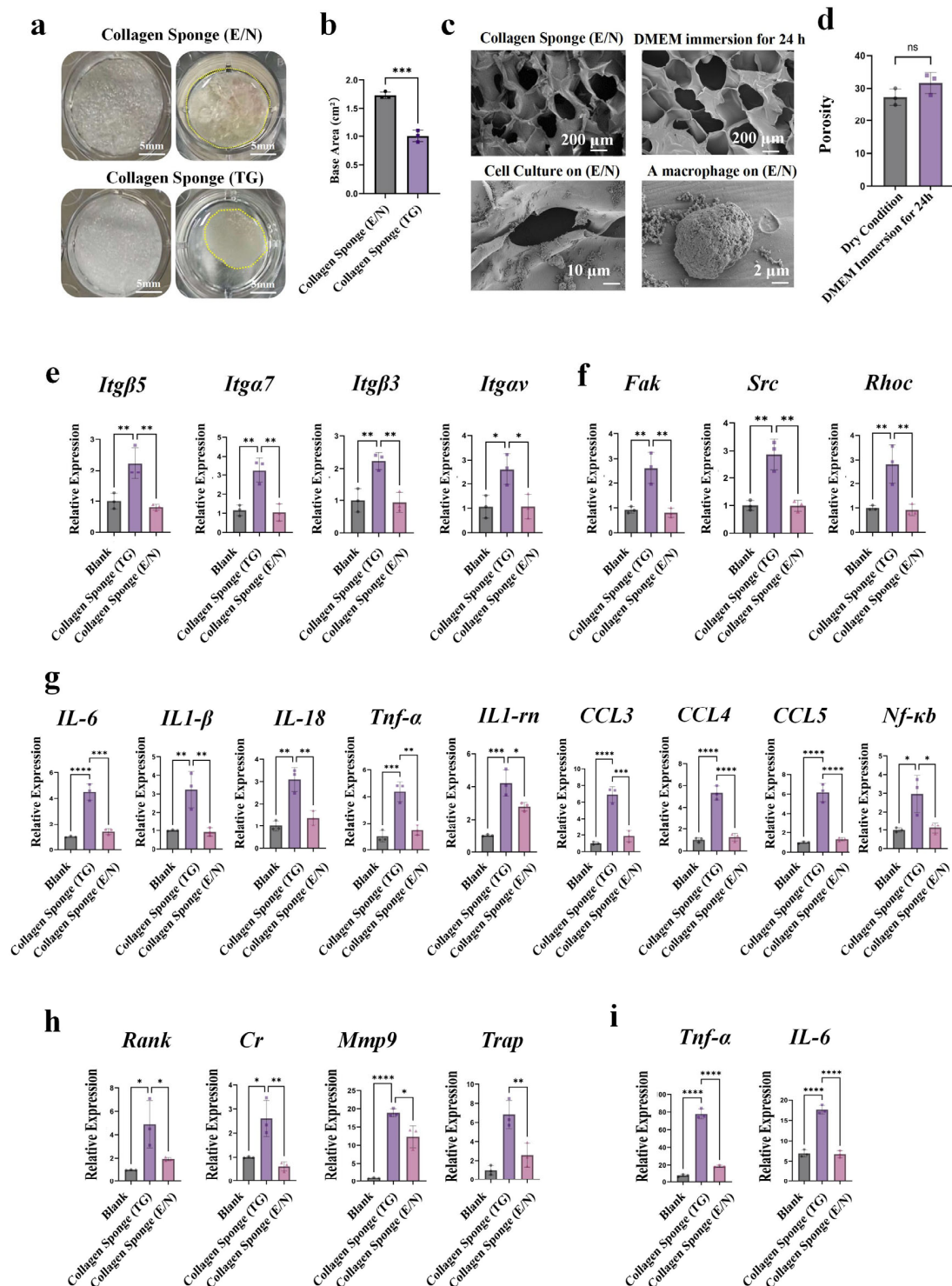


Figure 5. Collagen sponges with enhanced microstructural stability downregulated the Itgαvβ3/5-Src-RhoC-NF-κB axis and inflammatory response of macrophages. (a) Shrinkage status and (b) shrinkage rate of collagen sponge (TG) and collagen sponge (E/N) after hydration visualized by light microscopy ($n = 3$). (c) SEM images of pore structure changes in collagen sponges before and after hydration, and macrophage adhesion morphology. (d) Quantification of collagen sponge (E/N) Porosity under dry conditions and DMEM immersion for 24 h ($n = 3$) (E–H) qPCR analysis of genes associated with (e) integrins, (f) cytoskeletal organization, (g) inflammatory responses, and (h) osteoclast differentiation. (i) ELISA detection of TNF-α and IL-6. Data are presented as mean ± SD; statistical analysis was performed using unpaired two-tailed Student's t -test or one-way ANOVA followed by Tukey's multiple comparison post hoc test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

3.5. Conclusions

The present findings indicate that the reduced overall structural stability of collagen sponges, manifested as microstructural collapse, is closely associated with macrophage inflammatory activation through the integrin $\alpha v\beta 3/5$ –Src–RhoC–NF- κ B signaling axis. Under physiological-mimicking conditions, collagen scaffolds with insufficient structural stability undergo pronounced macroscopic shrinkage, leading to disruption of the porous framework and microstructural collapse. This structural deterioration correlates with the enhanced activation of integrins $\alpha v\beta 3$ and $\alpha v\beta 5$, the subsequent recruitment and phosphorylation of Src, the activation of downstream RhoC signaling, and the convergence on NF- κ B-dependent transcription of pro-inflammatory genes. In contrast, EDC/NHS cross-linked collagen scaffolds with improved mechanical stability exhibit minimal shrinkage, effectively preserving the microstructural integrity, maintaining low integrin activation, and thereby limiting downstream inflammatory signaling to sustain a basal low-inflammatory macrophage phenotype. These observations support a putative mechanotransduction axis linking scaffold structural instability, microstructural collapse, and macrophage inflammatory activation.

3.6. Limitations and Future Perspectives

This study has some limitations. This model reflects associative rather than definitive causal relationships, and direct validation at the protein level or through genetic perturbation of key signaling components will be required to confirm the proposed signaling sequence. All mechanistic investigations were performed using the RAW264.7 macrophage cell line, which, although widely used to model macrophage–material interactions [12,13,33,34], represents a simplified in vitro system. Accordingly, the inflammatory mechanisms described here should be interpreted within this experimental context. Future studies will incorporate primary macrophages, relevant in vivo models, and collagen scaffolds with graded cross-linking densities to further validate how cross-linking-mediated structural stability regulates the microstructural collapse and immune cell-associated inflammatory responses.

During the early stage of tissue regeneration, an appropriate level of inflammatory activation is required to recruit immune and regenerative cells. In contrast, the resolution of inflammation and the maintenance of a low-inflammatory microenvironment are essential during later stages to support tissue remodeling and regeneration. Accordingly, future collagen-modified regenerative scaffolds may need to possess tunable or responsive mechanical properties to accommodate the distinct material performance requirements across different regenerative phases. Therefore, developing collagen-based materials with precisely controllable mechanical properties represents an important direction for future research.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/bioengineering13020210/s1>, Figure S1: Transcriptomic analysis of macrophages cultured on collagen sponge; Table S1: List of Reagents Used in the Study; Table S2: Structural stability-related physicochemical properties of Collagen Sponges (TG and E/N) after 24 h immersion in DMEM.

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