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Novel composite bone cement modulates inflammatory response in vitro

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1. To evaluate the anti-inflammatory properties of enoxaparin sodium polymethylmethacrylate bone cement within the indirect co-culture model comprising endothelial cells and macrophages. 2. To investigate the impacts of inflammatory factors IL-6 and IL-10 on macrophage M2 polarisation and endothelial cell apoptosis. An indirect co-culture system of endothelial cells and macrophages was established by utilizing 1 µg/mL of lipopolysaccharide (LPS) to trigger an inflammatory response model. The experiment was categorized into 4 groups: blank control group, LPS-indicated group, PMMA + LPS group, and ES-PMMA + LPS group. Flow cytometry was performed to ascertain the apoptosis rate of endothelial cells and macrophage polarisation trend in the co-culture system. Meanwhile, ELISA, Western blotting, and immunofluorescence were adopted to measure the expression levels of Interleukin-6(IL-6), Tumour Necrosis Factor-α(TNF-α), Intercellular Cell Adhesion Molecule (ICAM), and Interleukin-10(IL-10) in cells and supernatants. In the detection of two typical polarisation proteins, CD86 and CD206, it was observed that the expression level of the CD86 protein, which indicates M1 polarisation, was elevated in the LPS-induced group in comparison to the blank control group (**$P < 0.01$). The expression level was found to be down-regulated in the ES-PMMA + LPS group (*$P < 0.05$). In contrast, the expression level of CD206 protein, which indicates the trend of M2-polarisation, was observed to be down-regulated in the LPS-induced group compared to the blank control group (***$P < 0.001$). Conversely, this expression level was up-regulated in the ES-PMMA + LPS group in comparison to the LPS-induced group (**$P < 0.01$). The expression of IL-6, TNF-α, IL-10, and ICAM was investigated in cell culture supernatants using the Elisa assay. The results showed that the LPS-induced group had higher levels of IL-6, TNF-α, and ICAM compared to the blank control group (***$P < 0.001$), while the LPS-induced group had lower levels of IL-10 (***$P < 0.001$). Additionally, the ES-PMMA + LPS-induced group showed lower levels of the aforementioned cytokines (**$P < 0.01$ or *$P < 0.05$) and higher levels of IL-10 (*$P < 0.05$). Western Blot and immunofluorescence results revealed that the expression of IL-6, TNF-α, and ICAM was up-regulated (***$P < 0.001$) and IL-10 was down-regulated (***$P < 0.001$) in the LPS-induced group compared with the blank control group. Compared with the LPS-induced group and PMMA + LPS group, in the ES-PMMA + LPS group, the expression of all three proteins was down-regulated (*$P < 0.05$ or **$P < 0.01$), whereas the expression of the IL-10 protein was up-regulated (***$P < 0.001$). The inflammatory proteins IL-6, TNF-α, and ICAM were shown to have higher fluorescence intensity in the LPS-induced group compared to the blank control group (***$P < 0.001$), the intensity of IL-10 was observed to be diminished (***$P < 0.001$). In contrast, the fluorescence intensity of IL-6, TNF-α, and ICAM was reduced in the ES-PMMA + LPS group relative to the LPS-induced group (***$P < 0.001$), the intensity of IL-10 was enhanced (***$P < 0.001$). In terms of endothelial cell apoptosis rate detection, the rate of apoptosis considerably reduced in the ES-PMMA + LPS-induced group when compared to the LPS-induced group (***$P < 0.001$) and rose noticeably in the LPS-induced group when compared to the blank control group (***$P < 0.001$). (1) In the co-culture system, ES-PMMA bone cement fulfills anti-inflammatory functions by impeding the expression of inflammatory factor IL-6 and promoting IL-10. (2) ES-PMMA bone cement facilitates the M2 polarisation response of macrophages and declines endothelial cell apoptosis within a co-culture system. (3) ES-PMMA bone cement can modify the local inflammatory environment by modulating the expression of inflammatory factors, which is potentially valuable for the application of cement-related surgery.

Abbreviations

ES	Enoxaparin sodium
PMMA	Polymethylmethacrylate
ES-PMMA	Enoxaparin Sodium-Polymethylmethacrylate
LPS	Lipopolysaccharide
IL-6	Interleukin-6
TNF-α	tumour necrosis factor-α
IL-10	Interleukin-10
ICAM	Intercellular Cell Adhesion Molecule
EC	Endothelial cell

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A central facet of the initiation of inflammation lies in the vascular response. Macrophages constitute the principal contributors to the inflammatory response and partake in the majority of pathological inflammatory processes within the body[1]. Stimulated by various physicochemical factors, macrophages rapidly begin to produce inflammatory cytokines, including endothelin, IL-6, and TNF-α[2]. This leads to the occurrence of an inflammatory response in the body. They have three main functions: antigen presentation, phagocytosis, and regulation of the immune system through the production of a range of cytokines and chemokine factors[3]. The characteristic M1/M2 phenotype is the two directions of macrophage polarisation, and studies have suggested that an imbalance in M1/M2 polarisation plays a key role in the inflammatory response and disease progression[4]. Macrophage and lymphocyte recruitment of endothelial cells from peripheral blood represents an early event in the development of vascular dysfunction[5]. Endothelial cells and macrophages are intimately involved in the regulation of inflammation, while endothelial cells induce selective macrophage growth, differentiation, and functional polarization[6,7].

It is well-known that the role of heparin and its derivatives on inflammation is receiving increasing attention from scholars[8]. Abbadi[9] et al. proved that heparin impeded the polarization of pro-inflammatory macrophages and promoted macrophage differentiation. Our prior studies have revealed that ES-PMMA bone cement possesses multiple properties as a novel type of material. Sun[10] et al. discovered that after carrying PMMA bone cement, enoxaparin sodium could be well released and did not affect its biological activity. Concurrently, Sang[11,12] et al. indicated that ES-PMMA bone cement could inhibit the role of CD40 in vascular endothelial cells and reduce local thrombosis. In terms of anti-inflammatory effects, our suggested that it induces macrophage polarisation towards the M2 phenotype and down-regulates the presence of the TLR4/NF-κB signaling pathway[13]. Moreover, ES-PMMA bone cement can exert local anti-inflammatory effects by modulating the inflammatory factors IL-6 and TNF-α[14]. However, in terms of anti-inflammation, ES-PMMA bone cement has yet to be thoroughly studied. Based on previous studies, we hypothesize that ES-PMMA reduces inflammatory cytokine release and promotes macrophage polarization towards the M2 phenotype, thereby mitigating endothelial cell apoptosis in co-culture. We recognized that single in vitro macrophages are customarily employed for studies of incurred inflammation, whereas the co-culture model appears to be a more efficacious portrayal of cell-cell interactions, given that it permits the unrestricted exchange of cytokines, nutrients, and other substances. It has been observed that the exosome MALAT1 from endothelial cells treated with oxidized low-density lipoprotein promotes macrophage M2 polarisation in a co-culture system[15]. Taking into account that endothelial cell-macrophage co-culture models might represent a more compelling approach to investigating inflammation. Hence, the objective of this study was to delve into the effects of ES-PMMA bone cement on macrophage polarisation response, endothelial cell apoptosis, and the inflammatory factors IL-6, TNF-α, ICAM, and IL-10. To this end, a co-culture model was employed to further explore the anti-inflammatory mechanisms of the new composite bone cement.

Materials and methods

Cell culture and treatment

Rat alveolar macrophages (PNS Life Sciences Co. Ltd. Wuhan, China) and rat inferior vena cava endothelial cells (PNS Life Sciences Co., Ltd. Wuhan, China) were cultured in RPMI Medium 1640(Gibco, USA) and the DMEM medium (Gibco, USA) was supplemented with 10% fetal bovine serum. The cells were cultured in a cell culture incubator maintained at 37 °C with 5% CO_2 for 2–3 days. The cell culture medium should be observed daily for changes in cell growth and colour. It is essential to renew the medium in time.

Co-culture and groups

The 8000 IU enoxaparin sodium powder (Chengdu Baiyu Co. Ltd., China) was premixed with 40 g of PMMA bone cement (Heraeus, Germany) and subsequently augmented with liquid[10]. Following the LPS-inducing concentrations mentioned in the references[14], we immersed both PMMA and ES-PMMA bone cement in cell complete medium at a ratio of 1 cm³/3 mL for 24 h and determined the final concentration of LPS to be 1 µg/ml. Subsequently, the two types of cells in the logarithmic growth phase were digested separately, counted, and prepared into cell suspensions. 2×10^5 macrophages and endothelial cells were separately seeded in 0.4 µm Transwell chambers. The medium of each group was replenished to 2 ml, and then the cells were collected for subsequent experiments after 24 h of continuous co-culture. (Fig. 1). The experiment was conducted in four distinct groups: a control group, an LPS-induced group, a PMMA + LPS group, and an ES-PMMA + LPS group.
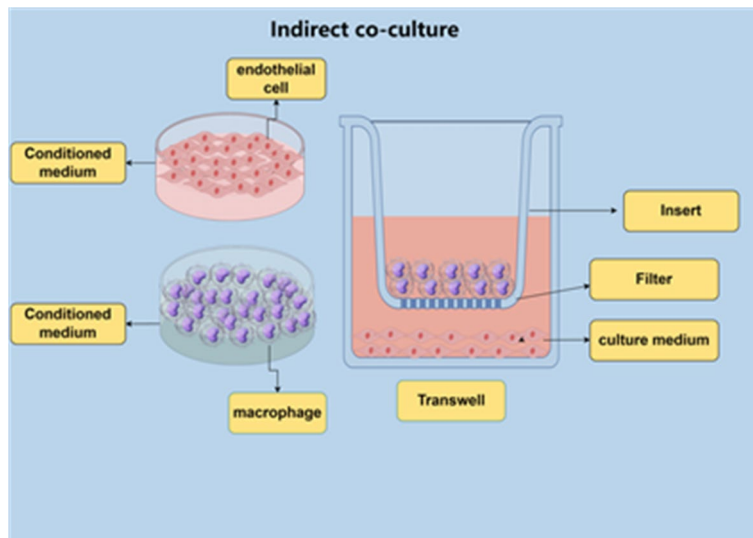
Fig. 1. Schematic diagram of co-culture modeling. Detection of inflammatory factors in an endothelial cell and macrophage co-culture system.

ELISA assay

The principal reagents utilized in the cellular experiments were the rat interleukin 6 (IL-6) ELISA kit (Elite, Wuhan, China No.E-EL-R0015c), the rat interleukin 10 (IL-10) ELISA kit (Elite, Wuhan, China No.E-EL-R0016c), the rat tumor necrosis factor-α (TNF-α) ELISA kit (Elite, Wuhan, China No.E-EL-R2856c), and the rat intercellular adhesion molecule 1 (ICAM-1/CD54) ELISA kit (Elite, Wuhan, China No.E-EL-R2850c). Refer to the kit instruction manual for operation. The 3rd generation cell culture supernatant was collected and centrifuged at 2000 rpm for 30 min to remove impurities and debris. Standard, blank, and sample wells were set up separately. Subsequently, 100 μl of the sample and standard were added to each well, and the plates were incubated for 90 min at 37 °C. Add TMB substrate to each well and incubate for 15 min in the dark. The reaction was terminated by the addition of 50 μl of termination solution to each well. : Finally, the optical density (OD) was assessed at 450 nm.

Western blotting

The protein content was quantified using the bicinchoninic acid (BCA) assay. The cells were grouped following the previously described methodology, and their original culture medium was aspirated and discarded. Following a single wash with 1× PBS, 1000 μL/well of RIPA (Solebro Technology Ltd, China) was added. Subsequently, the cell lysate was shaken at 4 °C for 30 min, after which it was transferred to a 1.5 mL centrifuge tube to obtain a cell protein sample. The protein concentration was determined by utilizing a standard curve constructed with the use of bovine serum albumin (BSA). The protein concentration of the samples was determined through the utilization of a Bradford assay. (A standard curve was plotted, with X representing protein concentration (in micromoles per milliliter) and Y representing final OD595 nm. This is based on the previously determined OD of the BSA standard, with the OD of the blank wells in the standard being deducted for the final reading. The protein concentration of the samples was then calculated from the standard curve and the dilution of the samples). Following the completion of the electrophoresis process, the gel was extracted and transferred to a membrane transfer buffer maintained at 4 °C. The gel was then transferred to a PVDF membrane. The primary antibodies, namely anti-IL-10 antibody (Abcam, UK), anti-ICAM antibody, and anti-IL-6 antibody (Abcam, UK), as well as anti-TNF-α antibody (Abcam, UK) (1:1000), were added to the incubation kit at 4 °C overnight under agitation. The membranes were incubated with HRP-labelled secondary antibody (goat anti-rabbit IgG; Abcam, UK) (1:8000) of the corresponding species origin for one and a 1.5 h at room temperature. They were then washed with TBST solution four times for 5 min each. ECL chemiluminescent reagents were used for color development. The protein markers utilized in the Western blotting procedure were sourced from Solebro Technology Ltd. (Beijing, China). Western blot analysis was conducted using a GAPDH antibody (Abcam, Cambridge, UK). Then, the films were scanned and imaged using an Epson Perfection V39 scanner, and the brightness values of the protein bands were analyzed. The method was to calculate the protein band brightness value ratio to the corresponding GAPDH band brightness value for each sample to obtain the corrected protein band brightness value. The control was used as the standard value1 and a bar graph was plotted.

Immunofluorescence

Cells were placed in 24-well plates, seeded overnight, and fixed by adding 1 mL of 4% paraformaldehyde fixative. Following a 60-minute incubation period with the blocking solution, the solution was removed and the cells were treated with the corresponding primary antibodies IL-10 (IL-10 monoclonal antibody; Invitrogen, USA) (1:100), ICAM (ICAM-1 polyclonal antibody protein tech, USA) (1:500), IL-6 (anti-IL-6 antibody rabbit product, Sigma, Germany) (1:50) and TNF-α (TNF Alpha monoclonal antibody protein tech, USA) (1:50) were

diluted overnight at 4 °C. Subsequently, the primary antibody was removed and the cells were washed 3 times with PBS solution for 10 min each time. A fluorescently labeled secondary antibody (goat anti-mouse IgG H&L; Abcam, UK) was added. The cells were incubated for 1 h at 37 °C. Fluorescence analysis was executed utilizing the ImageJ software.

Flow cytometry

Primary cultures of rat inferior vena cava endothelial cells were used (Hunan Fenghui Biotechnology Co. Ltd. China, for generations 2–3). The cultures were incubated in a 5% carbon dioxide-saturated humidity incubator at 37 °C for 2–3 days. Cells in the logarithmic growth phase were digested with trypsin (Sorabo Technology Co., Ltd., China) and inoculated into 6-well plates. The cells were grouped as described above and incubated for another 24 h. A total of 5×10^4 to 1×10^5 cells were collected and resuspended in binding buffer, as detailed in the Annexin V-FITC kit (Hangzhou Unitech Biotechnology Co. Ltd., China). Subsequently, 5 μl of Annexin V-FITC and 10 μl of propidium iodide (PI) solution were added. Subsequently, the cells were incubated at room temperature (20–25 °C) in the dark for a further 10–20 min, after which they were placed in an ice bath. The data were acquired using a FACS Calibur TM flow cytometer (BD Biosciences).

The expression of CD86 and CD206 was detected in each group using flow cytometry[16,17]. Rat alveolar macrophages (Punosai Life Sciences Co., Ltd. Wuhan China) were cultured in a complete medium (10% fetal bovine serum + 1% penicillin/streptomycin in RPMI 1640 medium) at 37 °C. The cells were digested to a growth density of 80% or more, counted, and 2×10^5 logarithmically growing macrophages were inoculated in 6-well plates with 3 replicate wells per group. Each tube of cells was resuspended with 100 μL PBS, and F4/80(Abcam, UK), CD86(Abcam, UK), and CD206(e-Bioscience, USA) antibodies were added, and incubated for 1 h at 4°C in the dark. The cells were resuspended in 400μL of PBS and analyzed using a flow cytometer. The results were analyzed using FlowJo l0 software.

Data analysis

The data were processed using GraphPad Prism 6. A one-way analysis of variance (ANOVA) was employed to conduct multiple comparisons between groups and calibrate the results with measurement data expressed as mean ± standard deviation (SD). The Tukey (HSD) test was applied to the post-hoc analysis test. Differences were considered statistically significant at *$P < 0.05$.

Results
Levels of secretion of the inflammatory factors in cultures were measured by ELISA

The expression of IL-6, TNF-α, IL-10, and ICAM in cell culture supernatants was detected by Elisa assay. It was demonstrated that the IL-6, TNF-α, and ICAM content LPS-induced group was increased (***$P < 0.001$) and IL-10 content was decreased (***$P < 0.001$) compared with the blank control group; while the group induced by ES-PMMA + LPS exhibited a decrease (***$P < 0.01$ or *$P < 0.05$) in the levels of the aforementioned cytokines compared to the LPS-induced group, except for IL-10, which showed an increase (*$P < 0.05$) (Fig. 2). ES-PMMA bone cement can restrain the secretion of pro-inflammatory factors IL-6, TNF-α, and ICAM, and stimulate the secretion of anti-inflammatory factor IL-10 to exert anti-inflammatory effects.
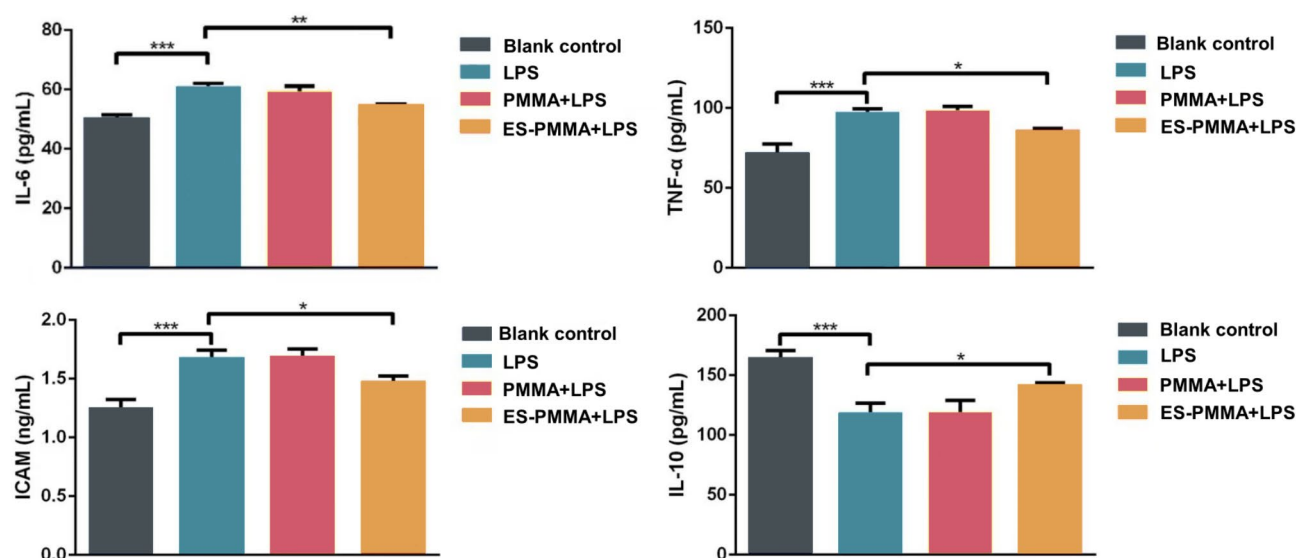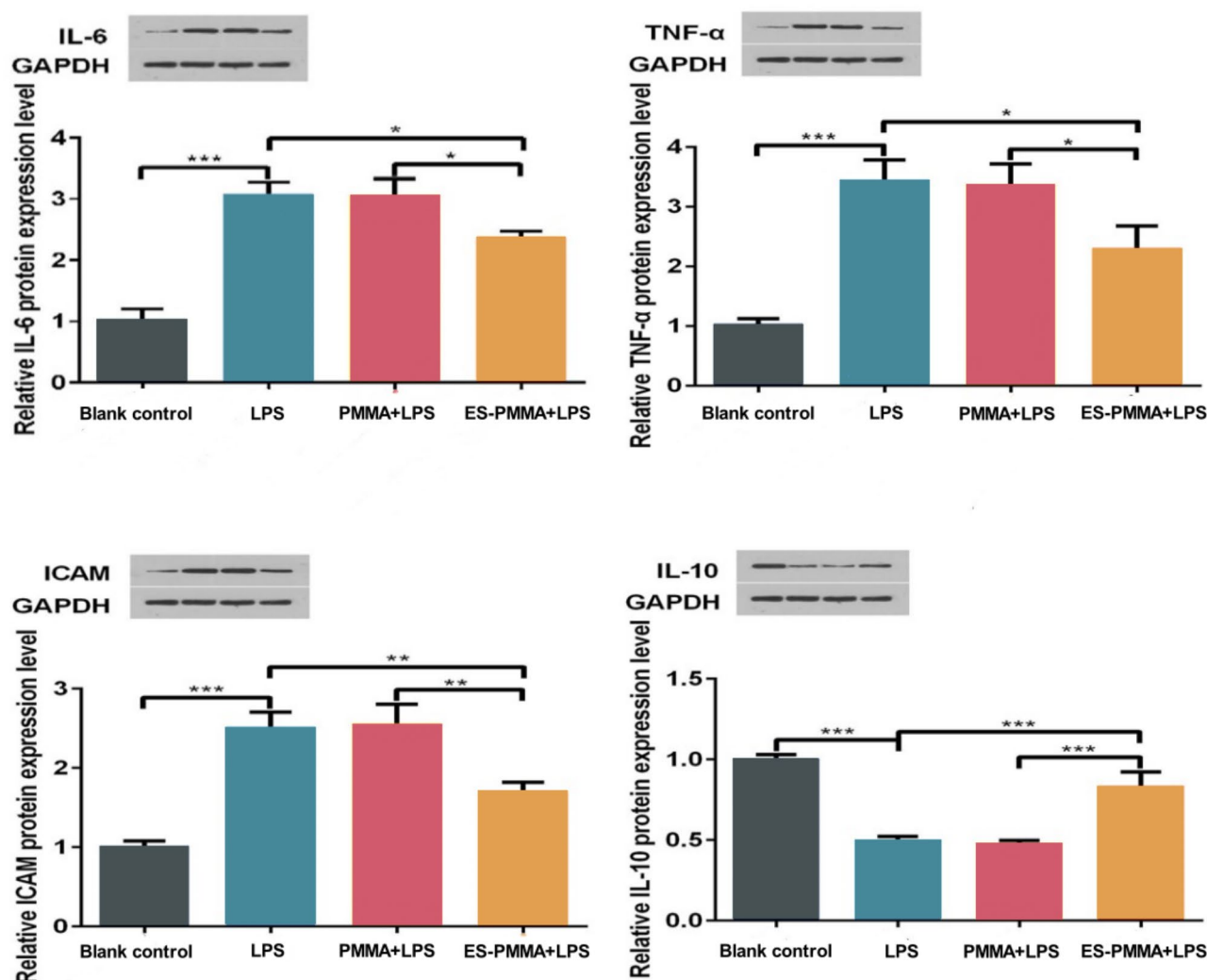

Fig. 2. ES-PMMA bone cement reduces the expression of pro-inflammatory factors in co-culture. Elisa assay for IL-6, TNF-α, ICAM and IL-10 content in the four groups of co-cultured cells resulted in an increase in IL-6, TNF-α, and ICAM factors and a decrease in IL-10 content in the LPS group compared with the blank control group (***$P < 0.001$); however, IL-6, TNF-α, and ICAM factors were decreased in the ES-PMMA group compared with the LPS group (*$P < 0.05$ or **$P < 0.01$) and increased IL-10 content (*$P < 0.05$) ($n = 3$).

Protein expression of IL-6, TNF-α, ICAM和IL-10 in cell cultures was determined by Western blot

Western Blot analysis disclosed that pro-inflammatory IL-6 and TNF-α proteins were released after endothelial cell injury, and the role of IL-6, TNF-α, and ICAM was up-regulated in the LPS-induced group compared with the blank control group (***$P < 0.001$); while the role of the three proteins was down-regulated in the ES-PMMA + LPS group relative to the LPS-induced and PMMA + LPS groups (*$P < 0.05$ or **$P < 0.01$).

In an inflammatory environment, macrophages in co-culture differentiated towards a pro-inflammatory phenotype, with IL-10 expression down-regulated in the LPS-induced group compared to the blank control group (***$P < 0.001$), whereas IL-10 protein expression was up-regulated in the ES-PMMA + LPS group compared to the LPS-induced group and the PMMA + LPS group (***$P < 0.001$) (Fig. 3). Western Blot experiments demonstrated that ES-PMMA bone cement could alter the inflammatory environment by up-regulating the expression of pro-inflammatory factor proteins and down-regulating anti-inflammatory factor proteins.

Fig. 3. ES-PMMA bone cement modulates the expression of pro/anti-inflammatory factors in co-culture by releasing enoxaparin sodium. In the co-culture system, inflammatory factors were determined by Western Blotting and GAPDH was used as an internal reference. The expression of IL-6, TNF-α, and ICAM was up-regulated in the LPS-induced group compared with the blank control group (***$P < 0.001$); the expression of all three proteins was down-regulated in the ES-PMMA + LPS group compared with the LPS-induced and PMMA + LPS groups (*$P < 0.05$ or **$P < 0.01$); the expression of IL-10 was down-regulated in the LPS-induced group compared with the blank control group (***$P < 0.001$), while IL-10 protein expression was up-regulated in ES-PMMA + LPS group compared with LPS-induced and PMMA + LPS groups (***$P < 0.001$).ES-PMMA had a significant advantage in regulating inflammatory protein expression. ($n = 3$).

Expression of inflammatory proteins in endothelial cells detected by Immunofluorescence

By immunofluorescence, we observed that in an indirect co-culture system of endothelial cells and macrophages, the fluorescence intensity of the inflammatory proteins IL-6, TNF-α, and ICAM was enhanced in the LPS-induced group compared with that of the blank control group (***$P < 0.001$), and the fluorescence intensity of IL-10 was decreased (***$P < 0.001$). In contrast, the fluorescence intensity of IL-6, TNF-α, and ICAM decreased in the ES-PMMA + LPS group compared with that in the LPS-induced group (***$P < 0.001$), and the fluorescence intensity of IL-10 increased (***$P < 0.001$). (Fig. 4) The results revealed that ES-PMMA bone cement in the co-culture system releases enoxaparin sodium, which regulates the expression of pro-inflammatory and anti-inflammatory factors and has anti-inflammatory activity.

Trends in endothelial cell apoptosis and macrophage polarisation by flow cytometry

The rate of endothelial cell apoptosis was markedly higher in the LPS-induced group compared to the blank control group (***$P < 0.001$). Whereas, in the ES-PMMA + LPS-induced group, the apoptosis rate decreased significantly compared to the LPS-induced group (***$P < 0.001$) (Fig. 5a). Meanwhile, in the detection of two representative polarisation proteins, CD86 and CD206, we found that the expression level of CD86 protein, which indicates the trend of M1 polarisation, was up-regulated in the LPS-induced group compared with the blank control group (**$P < 0.01$), while the expression level was down-regulated in the ES-PMMA + LPS group (*$P < 0.05$); the CD206 protein expression level, which indicates the trend of M2 polarisation, was down-regulated in the LPS-induced group compared with the blank control group (***$P < 0.001$), and up-regulated in the ES-PMMA + LPS group compared with the LPS-induced group (**$P < 0.01$) (Fig. 5b). The results demonstrated that ES-PMMA bone cement could promote the M2 phenotypic polarisation of macrophages and alter the direction of the inflammatory response, thereby reducing endothelial cell injury.

Discussion

Inflammation constitutes a defensive response elicited by the body in response to various physicochemical stimuli. It includes two major categories: infectious inflammation and non-infectious inflammation. Under normal circumstances, inflammation is typically a beneficial response and serves as the body's natural defense mechanism. Nevertheless, there are instances where inflammation can be harmful, such as when it targets transparent tissues or attacks the body's tissues. The factors that induce tissue damage, i.e. inflammatory agents, can be summarized in the following categories: biological factors like bacteria and viruses; physical factors such as temperature and radioactive substances; chemical factors, local necrotic tissues, and allergic reactions. Anti-inflammatory and pro-inflammatory factors are in balance under normal physiological conditions. In contrast, major orthopedic surgeries have the potential to instigate an inflammatory response within the body by prompting the release of prostaglandin, activating prostaglandin receptors, and augmenting the expression of inflammatory factors. Research has illustrated that numerous inflammatory changes can occur in the acute phase after joint replacement surgery[18], causing the release of inflammatory factors, including TNF-α, IL-6, IL-8, IL-1β, etc., which are not only signal transduction molecules but also effector molecules[19]. More worrisome is the fact that in patients with knee replacements, the inflammatory response induced by the surgery enhances this pathological change. The surgery itself is an invasive procedure with an attendant stress response, which includes the release of inflammatory factors, and thus the inflammatory state caused by the surgical stimulus can result in one of the most problematic post-operative complications for surgeons after knee surgery, "periprosthetic infection"[20,21].

Furthermore, PMMA bone cement is widely used in several clinical areas, and its advantages and disadvantages are well known to us[22,23]. During implantation of the bone cement, an inflammatory response can also be activated due to the release of heat during the polymerization reaction of the cement, as well as its inherent toxic effects It has been shown that PMMA bone cement inevitably triggers an immunoinflammatory response[24,25]. Hence, in light of a more comprehensive understanding of the physicochemical properties of PMMA bone cement, a growing volume of research has been conducted in recent years to optimize it. Han[26] et al. added 20% diatrizoate sodium to PMMA bone cement to obtain good radiopacity while maintaining its mechanical properties, setting properties, and biocompatibility. Carboxylic acid-functionalized polycarbonates have been demonstrated to enhance the antimicrobial properties of bone cement as an additive while preserving mechanical strength and cellular biocompatibility[27]. Given this, our team prepared a new bone cement material, ES-PMMA bone cement, and investigated a series of its properties[10–12]. By studying it we found that ES-PMMA bone cement has local antithrombotic and anti-inflammatory biological properties, and at the same time, it showed some osteogenic capacity after the addition of alendronate[28]. All of these indicate that it has a promising research value and potential. Thus, in the subsequent study, we further illustrated its in vitro anti-inflammatory properties by employing an indirect co-culture method with endothelial cells and macrophages.

Heparin is known to exhibit various biological activities beyond its anticoagulant effects. Among these, it is especially worth mentioning its anti-inflammatory properties, its ability to promote the release of pancreatic lipase and hepatic lipase, and its ability to reduce angiogenesis[29–31]. In recent years, the co-cultivation system has also garnered significant attention from scholars. Liu et al. Natural history of atherosclerosis using a co-culture model of endothelial cells, macrophages, and vascular smooth muscle cells[32]. Miki[33] and others have also found that co-cultures are superior to single-cell cultures in mimicking the in vivo environment.

In a previous study, we performed a preliminary characterization of the anti-inflammatory effects of ES-PMMA in a single-cell culture model[14]. In the co-culture model of this study, the ES-PMMA + LPS group of bone cement decreased the expression of pro-inflammatory factors IL-6 and TNF-α and promoted the expression of anti-inflammatory factor IL-10 relative to the LPS-induced and PMMA + LPS groups. We were aware that IL-6, TNF-α, and IL-10 are often used for inflammation studies. It has been shown[34] that Ligustrazine inhibits the inflammatory response of human endometrial stromal cells via the STAT3/IGF2BP1/RELA axis. Sun[35] et al. also
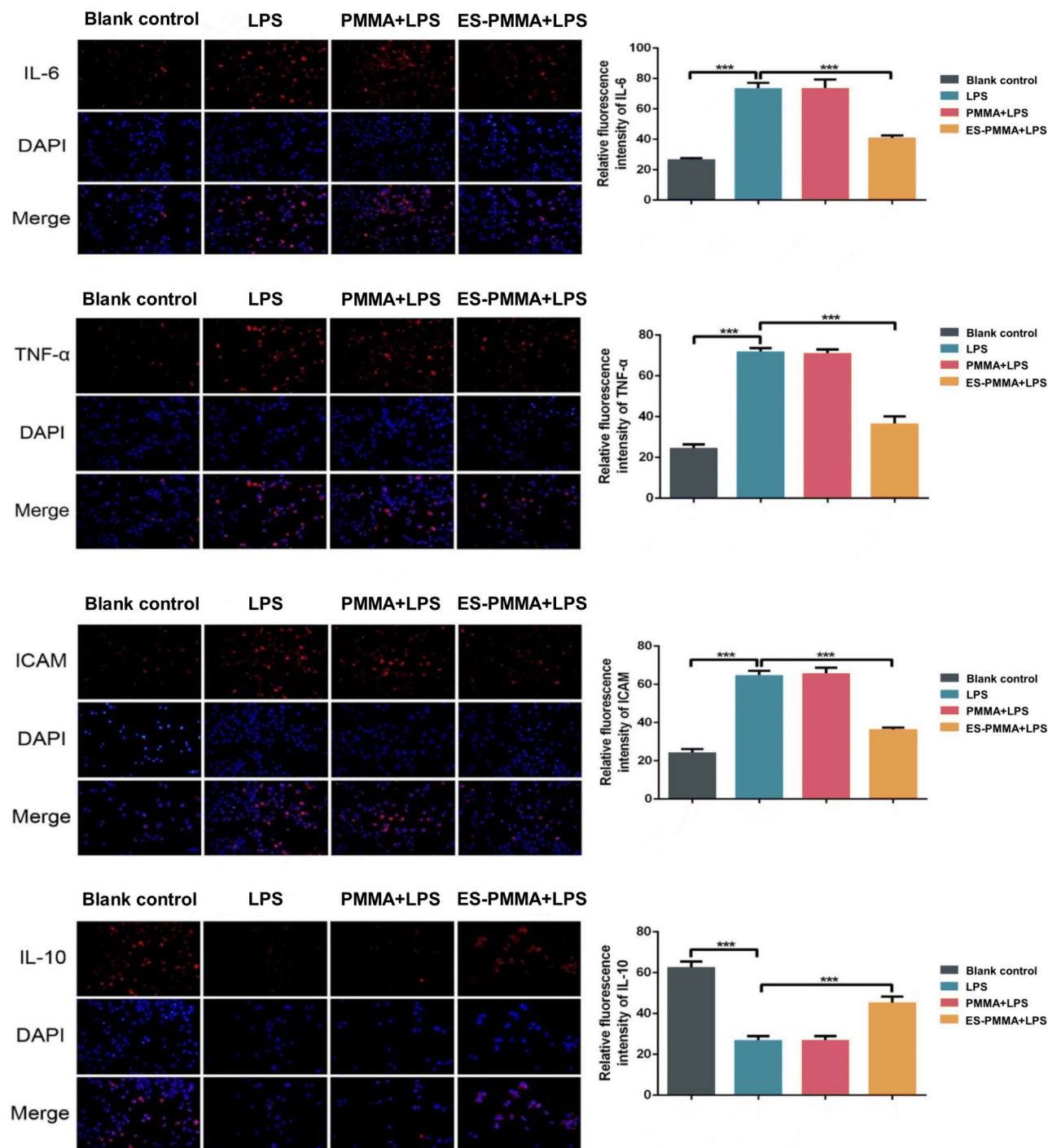
Fig. 4. Changes in fluorescence intensity of ES-PMMA bone cement affecting inflammatory factors in a co-culture system. By immunofluorescence we found that in the co-culture system of the two cells, the fluorescence intensity of the inflammatory proteins IL-6, TNF-α and ICAM was increased in the LPS-induced group compared to the blank control group (***$P < 0.001$), and the fluorescence intensity of IL-10 was reduced (***$P < 0.001$); in contrast, the fluorescence intensity of the IL-6, TNF-α, and ICAM was reduced in the ES-PMMA + LPS group compared to the LPS-induced group (***$P < 0.001$); and the fluorescence intensity of the IL-10 was increased (***$P < 0.001$). This indicates that ES-PMMA bone cement in the co-culture system has anti-inflammatory potency. This indicates that ES-PMMA bone cement in the co-culture system has anti-inflammatory potency. ($n = 3$).
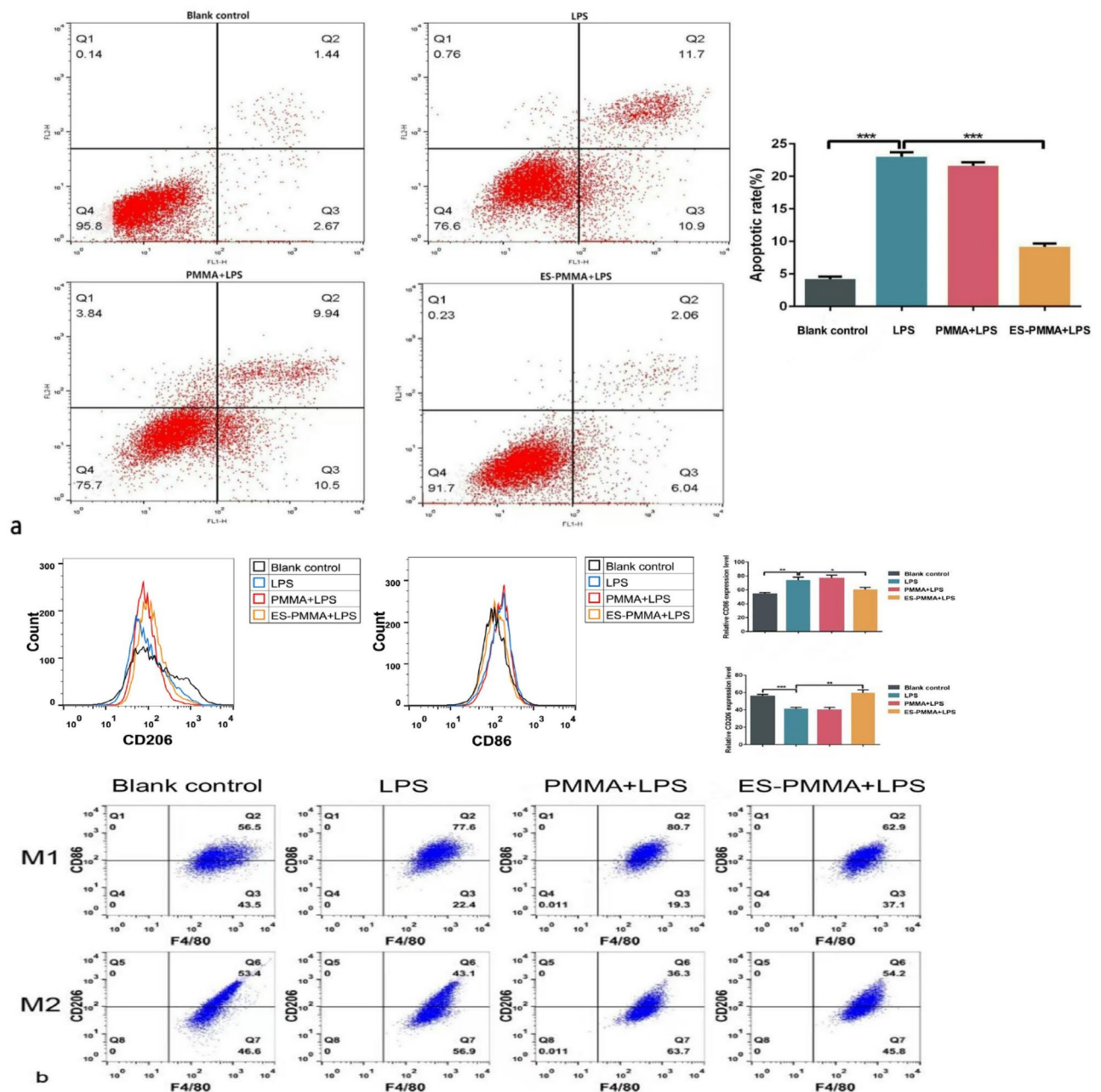
Fig. 5. Flow cytometric detection of endothelial cell apoptosis rates and macrophage polarisation trends in co-culture systems. (**a**) The apoptosis rate increased significantly in the LPS-induced group compared with the blank control group (***$P < 0.001$) and decreased significantly in the ES-PMMA + LPS-induced group compared with the LPS-induced group (***$P < 0.001$). (**b**) There are many specific proteins for macrophage polarisation, in the detection of two representative polarisation proteins, CD86 and CD206, we found that the expression level of CD86 protein, which indicates the trend of M1 polarisation, was up-regulated in the LPS-induced group compared to the blank control group (**$P < 0.01$), while the expression level was down-regulated in the ES-PMMA + LPS group (*$P < 0.05$); the expression level of CD206 protein expression level was down-regulated in the LPS-induced group compared with the blank control group (***$P < 0.001$) and up-regulated in the ES-PMMA + LPS group compared with the LPS-induced group (**$P < 0.01$). The fluorescence of CD206 markers representing M2 polarisation in the histogram was higher in the ES-PMMA + LPS group than in the other experimental groups. The results indicated that ES-PMMA bone cement could promote the M2 phenotype polarisation of macrophages. ($n = 3$).

found that B-cell-derived IL-10 is critical for the resolution of LP-induced acute lung injury and may serve as a potential therapeutic target. ES-PMMA bone cement can exert anti-inflammatory effects by modulating these inflammatory factors, which is consistent with literature reports.

Macrophages can produce various inflammatory mediators in autoimmune and autoinflammatory diseases. Depending on the environment in which the organism lives, macrophages present in different tissues polarise to form different macrophage subtypes, including both M1 and M2 types[36]. The microbial component lipopolysaccharide (LPS) can drive macrophage polarisation towards the M1 phenotype, which has a pro-inflammatory response and produces inflammation-associated factors such as IL-6, IL-12 and tumour necrosis factor (TNF), as opposed to the M2 macrophage, which has an anti-inflammatory response and the ability to repair damaged tissue[37,38]. In an inflammatory environment, macrophages are first polarised to a pro-inflammatory M1-type phenotype to help the host fight pathogens. As inflammation subsides, macrophages are polarised to form an anti-inflammatory response of the M2-type phenotype to repair tissue damage.Central to macrophage polarization are alterations in the expression of cell surface markers. These markers play a major role in tissue repair, angiogenesis, and metabolism[39]. Luo M[40] et al. identified an important role for macrophage polarisation in inflammatory diseases. In the detection of two macrophage-specific proteins, CD86 and CD806, we found that the expression levels of CD86, TNF-α, and IL-6 were significantly lower in the ES-PMMA + LPS group than in the LPS-induced group; meanwhile, the expression levels of CD206 and IL-10 were increased compared with the LPS + PMMA group and the LPS-induced group. Compared with conventional PMMA bone cement, ES-PMMA bone cement induced M2 phenotypic polarisation of macrophages and increased secretion of anti-inflammatory factors, which was associated with the anti-inflammatory effect of enoxaparin sodium.

It has been reported to demonstrate that endothelial cells can be activated and are capable of actively recruiting effector immune cells during the inflammatory response and that cytokines such as IL-6, IL-1β and tumour necrosis factor (TNF) induce endothelial cell activation, but also through pathogen-associated molecular patterns such as lipopolysaccharides[41–43]. Chen et al.[44] reported that exosomal lncRNA growth arrest-specific 5 regulates apoptosis of macrophages and vascular endothelial cells in atherosclerosis. Whereas tissue injury causes immediate activation of the inflammatory cascade, activated intertissued macrophages release inflammatory mediators (TNF-α, IL-1β, IL-6)[45]. For the endothelial cell apoptosis assay we found that PMMA bone cement promoted LPS-induced endothelial cell apoptosis, which exacerbated the inflammatory response with consequent macrophage M1-type polarisation after the release of inflammatory factors from endothelial cell apoptosis. Conversely, ES-PMMA inhibited the apoptotic response of endothelial cells and promoted M2-type polarisation of macrophages to protect endothelial cells.

ICAM-1 can be expressed on a wide range of cells such as endothelial cells, fibroblasts, and most leukocyte subpopulations[46–48]. Inflammatory factors such as IL-1, and TNF-α can increase ICAM-1 expression in multiple cell types[46,49]. In response to inflammatory stimuli, ICAM-1 was upregulated not only on resident cells but also on macrophages[50,51]. Notably, in our study, we noticed that ICAM protein expression was reduced in the ES-PMMA + LPS-induced group compared with the PMMA + LPS-induced and LPS-induced groups, which could also indicate that ES-PMMA bone cement inhibits the exacerbation of inflammatory response by reducing the expression of ICAM protein after endothelial injury, and the recruitment of macrophages.

Concerning studies on the anti-inflammatory effects of bone cement, it has been shown that Injectable bone cement with magnesium-containing microspheres enhances osteogenesis via anti-inflammatory immunoregulation[52]. Meantime, our research has some limitations, and the in vitro model may not be able to fully replicate the complex in vivo environment, and we will further investigate it in animals in future studies. Future studies should incorporate in vivo models to validate the anti-inflammatory efficacy of ES-PMMA under physiological conditions.

Conclusion

In the present study, we simulated the in vivo inflammatory environment using an indirect co-culture model of endothelial cells and macrophages and found that ES-PMMA exerts anti-inflammatory effects by inhibiting the pro-inflammatory factors IL-6, TNF-α, and ICAM, and promoting the expression of the anti-inflammatory factor IL-10, which complements the biological properties of this novel composite material very well, and also provides certain theoretical basis. These findings provide a foundation for future clinical studies exploring the use of ES-PMMA in reducing postoperative inflammation and improving implant longevity.

Data availability

The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

References

1. Hamidzadeh, K., Christensen, S. M., Dalby, E., Chandrasekaran, P. & Mosser, D. M. Macrophages and the recovery from acute and chronic inflammation. *Annu. Rev. Physiol.* **79** (1), 567–592. https://doi.org/10.1002/abio.370040210 (2017).
2. Shi, M. et al. Europium-doped mesoporous silica nanosphere as an immune-modulating osteogenesis/angiogenesis agent.[J]. Biomaterials,2017,176–187. https://doi.org/10.1016/j.biomaterials.2017.08.027
3. Fujiwara, N. & Kobayashi, K. Macrophages in inflammation. *Curr. Drug Targets Inflamm. Allergy*. **4** (3), 281–286. https://doi.org/10.2174/1568010054022024 (2005).
4. Kim, H. et al. Exosome-guided phenotypic switch of M1 to M2 macrophages for cutaneous wound healing. Adv Sci (Weinh). ;6(20):1900513. https://doi.org10.1002/advs.201900513. (2019).

5. Xu, Y. et al. Blockade of PKC-beta protects HUVEC from advanced glycation end products induced inflammation. *Int. Immunopharmacol.* 10 (12), 1552–1559. https://doi.org/10.1016/j.intimp.2010.09.006 (2010).

6. Privratsky, J. R. et al. A macrophage-endothelial immunoregulatory axis ameliorates septic acute kidney injury.[J]. *Kidney Int.* 3, 514–528. https://doi.org/10.1016/j.kint.2022.10.008 (2023).

7. Medrano-Bosch, M. et al. Monocyte-endothelial cell interactions in vascular and tissue remodeling. *[J] Front. Immunol.* ,2025,1196033. https://doi.org/10.3389/fimmu.2023.1196033

8. Hogwood, J., Mulloy, B., Lever, R., Gray, E. & Page, C. P. Pharmacology of heparin and relateddrugs: an update. *Pharmacol. Rev.* 75 (3), 328–379. https://doi.org/10.1124/pharmrev.122.000684 (2023).

9. Abbadi, A. et al. Heparin inhibits pro-inflammatory and promotes anti-inflammatory macrophage polarization under hyperglycemic stress. *J. Biol. Chem.* 295 (15), 4849–4857. https://doi.org/10.1074/jbc.RA119.012419 (2020).

10. Sun, H. et al. Release characteristics of Enoxaparin sodium-loaded polymethylmethacrylate bone cement. *J. Orthop. Surg. Res.* 16 (1), 108. https://doi.org/10.1186/s13018-021-02223-w (2021).

11. Sang, L. et al. The mechanism by which Enoxaparin sodium-high-viscosity bone cement reduces thrombosis by regulating CD40 expression in endothelial cells. *BMC Musculoskelet. Disord*. 23 (1), 513. https://doi.org/10.1186/s12891-022-05469-5 (2022).

12. Sang, L. et al. LncRNA MSTRG.22719.16 mediates the reduction of Enoxaparin sodium high-viscosity bone cement-induced thrombosis by targeting the ocu-miR-326-5p/CD40 axis. *J. Orthop. Surg. Res.* 18, 716. https://doi.org/10.1186/s13018-023-04109-5 (2023).

13. Fan, W. et al. Enoxaparin sodium bone cement plays an anti-inflammatory Immunomodulatory role by inducing the polarization of M2 macrophages. *J. Orthop. Surg. Res.*, 2023,1:380. https://doi.org/10.1186/s13018-023-03865-8

14. Hao, K. et al. Enoxaparin sodium bone cement displays local anti-inflammatory effects by regulating the expression of IL-6 and TNF-α.[J]. *Heliyon* 6, e16530. https://doi.org/10.1016/j.heliyon.2023.e16530 (2023).

15. Huang, C. et al. Exosomal MALAT1 derived from oxidized low-density lipoprotein-treated endothelial cells promotes M2 macrophage polarization. [J]. *Mol. Med. Rep. 2018*, 1:509–515. https://doi.org/10.3892/mmr.2018.8982

16. Shi, F. et al. Tumor-associated macrophages in direct contact with prostate cancer cells promote malignant proliferation and metastasis through NOTCH1 pathway. [J]. *Int. J. Biol. Sci. 2024*, 16:5994–6007. https://doi.org/10.7150/ijbs.73141

17. Almendros, I. et al. Intermittent hypoxia-induced changes in tumor-associated macrophages and tumor malignancy in a mouse model of sleep apnea[. *J] Am. J. Respir Crit. Care Med. 2014*, 5:593–601. https://doi.org/10.1164/rccm.201310-1830OC

18. Wasko, M. K. et al. Neutrophil-to-lymphocyte ratio shows faster changing kinetics than C-reactive protein after total hip and knee arthroplasty. [J]. *J. Orthop. Translat*, 2017, 36–41. https://doi.org/10.1016/j.jot.2017.05.008

19. Tichelaar, Y. I., Kluin-Nelemans, H. J. & Meijer, K. Infections and inflammatory diseases as risk factors for venous thrombosis. A systematic review. [J]. Thromb Haemost,2012,5:827 – 37. https://doi.org/10.1160/TH11-09-0611

20. Wasko, M. K. et al. Measurement of the inflammatory response in the early postoperative period after hip and knee arthroplasty. [J]. *Clin. Chem. Lab. Med.* 11, 1785–1792. https://doi.org/10.1515/cclm-2014-1055 (2015).

21. Fontalis, A. et al. Inflammatory response in Robotic-Arm-Assisted versus conventional Jig-Based TKA and the correlation with early functional outcomes: results of a prospective randomized controlled trial. *J. Bone Joint Surg. Am.* 104 (21), 1905–1914. https://doi.org/10.2106/JBJS.22.00167 (2022).

22. O'Dowd-Booth, C. J. et al. Bone cement: perioperative issues, orthopedic applications, and future developments. *[J] J. Perioper Pract. 2011*, 9:304–308. https://doi.org/10.1177/175045891102100902

23. Garnon, J. et al. PMMA bone cement in interventional Oncology.[J]. *Crit. Rev. Biomed. Eng. 2024*, 1:35–50. https://doi.org/10.1615/CritRevBiomedEng.2021037591

24. de Melo Carpaneda, E. & Carpaneda, C. A. Adverse results with PMMA fillers. [J]. Aesthetic Plast Surg,2012,4:955 – 63. https://doi.org/10.1007/s00266-012-9871-8

25. Yamanaka, Y., Karuppaiah, K. & Abu-Amer, Y. Polyubiquitination events mediate polymethylmethacrylate (PMMA) particle activation of NF-kappaB pathway. [J]. *J. Biol. Chem. 2011*, 27:23735–23741. https://doi.org/10.1074/jbc.M111.223669

26. Han, J. et al. Modification and evaluation of diatrizoate sodium containing polymethyl methacrylate bone cement. [J]. *J. Biomater. Appl. 2023*, 8853282221150359. https://doi.org/10.1177/08853282221150359

27. Liang, Z. C. et al. Carboxylic acid-functionalized polycarbonates as bone cement additives for enhanced and sustained release of antibiotics. [J]. *J. Control Release*, 1970,:871–881. https://doi.org/10.1016/j.jconrel.2020.10.018

28. Xiao, Z. et al. Bone healing study of alendronate combined with Enoxaparin sodium bone cement in rabbits with bone defects. [J]. *J. Orthop. Surg. Res.* ,1970,1:431. https://doi.org/10.1186/s13018-022-03330-y

29. Li, L. F. et al. Low-Molecular-Weight heparin reduces Ventilation-Induced lung injury through hypoxia inducible Factor-1α in a murine endotoxemia model. *Int. J. Mol. Sci.* 21 (9), 3097. https://doi.org/10.3390/ijms21093097 (2020).

30. Zhao, D. et al. Heparin rescues sepsis-associated acute lung injury and lethality through the suppression of inflammatory responses. *Inflammation* 35 (6), 1825–1832. https://doi.org/10.1007/s10753-012-9503-0 (2012).

31. Morris, A. et al. The role of heparanase in pulmonary cell recruitment in response to an allergic but not non-allergic stimulus. *PLoS One.* 10 (6), e0127032. https://doi.org/10.1371/journal.pone.0127032 (2015).

32. Liu, M. et al. Co-culture models of endothelial cells, macrophages, and vascular smooth muscle cells for the study of the natural history of atherosclerosis. *PLoS One.* 18 (1), e0280385. https://doi.org/10.1371/journal.pone.0280385 (2023).

33. Miki, Y. et al. The advantages of co-culture over mono cell culture in simulating in vivo environment. *J. Steroid Biochem. Mol. Biol.* 131 (3–5), 68–75. https://doi.org/10.1016/j.jsbmb.2011.12.00 (2012).

34. Feng, Y., Dong, H. & Zheng, L. Ligustrazine inhibits inflammatory response of human endometrial stromal cells through the STAT3/IGF2BP1/RELA axis. [J]. *Pharm. Biol. 2023*, 1:666–673. https://doi.org/10.1080/13880209.2023.2195883

35. Sun, Z., Chen, A. & Fang, H. B cell-derived IL-10 promotes the resolution of lipopolysaccharide-induced acute lung injury. *Cell. Death Dis.* 14 (7), 418. https://doi.org/10.1038/s41419-023-05954-2 (2023).

36. Martinez, F. O. & Gordon, S. The M1 and M2 paradigm of macrophage activation: time for reassessment. [J]. F1000Prime Rep, 2024,:13. https://doi.org/10.12703/P6-13

37. Xiao, Y. et al. GCH1 reduces LPS-induced alveolar macrophage polarization and inflammation by Inhibition of ferroptosis. *[J] Inflamm. Res.* ,2023,10–11:1941–1955. https://doi.org/10.1007/s00011-023-01785-1

38. Luo, J. et al. Nrf2 deficiency exacerbated CLP-Induced pulmonary injury and inflammation through Autophagy- and NF-κB/PPARγ-Mediated macrophage polarization. *Cells* 11 (23), 3927. https://doi.org/10.3390/cells11233927 (2022).

39. Van Dyken, S. J. & Locksley, R. M. Interleukin-4- and interleukin-13-mediated alternatively activated macrophages: roles in homeostasis and disease. [J]. *Annu. Rev. Immunol.* https://doi.org/10.1146/annurev-immunol-032712-095906 (2013). 317 – 43.

40. Luo, M. et al. Macrophage polarization: an important role in inflammatory diseases.[J]. Front Immunol,2025,1352946. https://doi.org/10.3389/fimmu.2024.1352946

41. Georganaki, M., van Hooren, L. & Dimberg, A. Vascular targeting to increase the efficiency of immune checkpoint Blockade in cancer. [J]. Front Immunol,2018, 3081. https://doi.org/10.3389/fimmu.2018.03081

42. Amersfoort, J., Eelen, G. & Carmeliet, P. Immunomodulation by endothelial cells-partnering up with the immune system?[J]. *Nat. Rev. Immunol. 2022*, 9:576–588. https://doi.org/10.1038/s41577-022-00694-4

43. Hellenthal, K. E. M., Brabenec, L. & Wagner, N. M. Regulation and dysregulation of endothelial permeability during systemic inflammation. [J]. Cells,1970,12: https://doi.org/10.3390/cells11121935

44. Chen, L. et al. Exosomal LncRNA GAS5 regulates the apoptosis of macrophages and vascular endothelial cells in atherosclerosis. [J]. *PLoS One 2024*, 9: e0185406. https://doi.org/10.1371/journal.pone.0185406

45. Cai, S. et al. Mitochondrial dysfunction in macrophages promotes inflammation and suppresses repair after myocardial infarction. [J]. *J. Clin. Invest.* https://doi.org/10.1172/JCI159498 (2022).
46. Woodfin, A. et al. ICAM-1-expressing neutrophils exhibit enhanced effector functions in murine models of endotoxemia. *[J] Blood 2016*, **7**:898–907. https://doi.org/10.1182/blood-2015-08-664995
47. Proebstl, D. et al. Pericytes support neutrophil subendothelial cell crawling and breaching of venular walls in vivo. [J]. *J. Exp. Med. 2012*, **6**:1219–1234. https://doi.org/10.1084/jem.20111622
48. Springer, T. A. Adhesion receptors of the immune system. [J]. Nature,1990,6283:425 – 34. https://doi.org/10.1038/346425a0
49. Hubbard, A. K. & Rothlein, R. Intercellular adhesion molecule-1 (ICAM-1) expression and cell signaling cascades. [J]. *Free Radic Biol. Med.* **9**, 1379–1386. https://doi.org/10.1016/s0891-5849(00)00223-9 (2000).
50. Wiesolek, H. L. et al. Intercellular adhesion molecule 1 functions as an efferocytosis receptor in inflammatory macrophages. *Am. J. Pathol.* **190** (4), 874–885. https://doi.org/10.1016/j.ajpath.2019.12.006 (2020).
51. Yang, M. et al. ICAM-1 suppresses tumor metastasis by inhibiting macrophage M2 polarization through Blockade of efferocytosis. *Cell. Death Dis.* **6** (6), e1780. https://doi.org/10.1038/cddis.2015.144 (2015).
52. Tan, S. et al. Injectable bone cement with magnesium-containing microspheres enhances osteogenesis via anti-inflammatory immunoregulation. *[J] Bioact Mater.* **10**, 3411–3423. https://doi.org/10.1016/j.bioactmat.2021.03.006 (2021).

Author contributions

Kangning Hao, Jie Hu for Methodology, Validation and investigation. Kangning Hao and Jiangyong Wang for the writing-original draft, data curation, resources, and formal analysis. Fei Li for conceptualization and writing-review & editing and Supervision. Fei Li is responsible for project administration and funding acquisition.

Funding

Declarations

Ethics approval and consent to participate

All experiments were approved by the Ethics Committee of the Third Hospital of Shijiazhuang City, Hebei Province, China. Address: No.15, Ti Yu South Street, Shijiazhuang City, Hebei Province, China. (number: 2018-006).

Competing interests

The authors declare no competing interests.

Additional information

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