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Study of the immunologic response of marine-derived collagen and gelatin extracts for tissue engineering applications

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ABSTRACT

The host immunologic response to a specific material is a critical aspect when considering it for clinical implementation. Collagen and gelatin extracted from marine sources have been proposed as biomaterials for tissue engineering applications, but there is a lack of information in the literature about their immunogenicity. In this work, we evaluated the immune response to collagen and/or gelatin from blue shark and codfish, previously extracted and characterized. After endotoxin evaluation, bone marrow-derived macrophages were exposed to the materials and a panel of pro- and anti-inflammatory cytokines were evaluated both for protein quantification and gene expression. Then, the impact of those materials in the host was evaluated through peritoneal injection in C57BL/6 mice. The results suggested shark collagen as the less immunogenic material, inducing low expression of pro-inflammatory cytokines as well as inducible nitric oxide synthase (encoded by *Nos2*) and high expression of Arginase 1 (encoded by *Arg1*). Although shark gelatin appeared to be the material with higher pro-inflammatory expression, it also presents a high expression of IL-10 (anti-inflammatory cytokine) and Arginase (both markers for M2-like macrophages). When injected in the peritoneal cavity of mice, our materials demonstrated a transient recruitment of neutrophil, being almost non-existent after 24 hours of injection. Based on these findings, the studied collagenous materials can be considered interesting biomaterial candidates for regenerative medicine as they may induce an activation of the M2-like macrophage population, which is involved in suppressing the inflammatory processes promoting tissue remodeling.

Statement of significance

Marine-origin biomaterials are emerging in the biomedical arena, namely the ones based in marine-derived collagen/gelatin proposed as cell templates for tissue regeneration. Nevertheless, although the major cause of implant rejection in clinical practice is the host's negative immune response, there is a lack of information in the literature about the immunological impact of these marine collagenous materials. This work aims to contribute with knowledge about the immunologic response to collagen/gelatin extracted from blue shark and codfish skins. The results demonstrated that despite some differences observed, all the materials can induce a macrophage phenotype related with anti-inflammation resolution and then act as immuno-modulators and anti-inflammatory inducible materials.

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1. Introduction

The origin of name collagen derives from the Greek words for "glue" and "to produce" and was consider, in the past, as the glue that holds cells in place [1,2]. Collagen is a complex and vital protein that represents between 25 to 35% of total protein content in vertebrates [3,4]. This protein can be found in fibrous and connective tissues such ligaments, tendons, muscles and skin, as well as in cornea, bones, cartilage, blood vessels and in interstitial tissues of all parenchymal organs [5,6]. It is the principal structural protein of extracellular matrix (ECM) giving support and mechanical stability and therefore, playing a key role in embryogenic development and tissue regeneration [7]. Due to all of this, collagen is one of the central mainstays of biological structure in all animals. Type I stands out as the major type of collagen, accounting for 80 – 90% of the total collagen present in the body being abundant in skin, bones, tendon, and cornea [2,8].

Different materials such collagen peptides (resulting from proteolysis) or gelatin, can be derived from collagen by means of physical, chemical or thermal treatments. Gelatin is the resulting product from the thermal degradation of native collagen protein. The thermal treatment leads to the destruction of cross-linkages between the polypeptide chains along with some breakage of polypeptide bonds. That generate a partial hydrolysate and water-soluble form of the original protein with different molecular weights (MWs) [9].

Collagen and gelatin are commonly extracted from mammal by-products such skin, bones, and cartilage from porcine or bovine origin. Such resources are, many times, associated with high-cost production, high risk of immunogenicity associated to allergies [10–12], zoonosis transmission, outbreak of infectious diseases derived from prions or virus eventually present in mammal materials, such as bovine spongiform encephalopathy (BSE), transmissible spongiform encephalopathy (TSE), and foot and mouth disease (FMD) [13–15], as well as religious constraints (kosher and halal). In an attempt to find a safe alternative to mammalian collagen, marine origin by-products arise as a promising alternative since there are no known diseases that could be transmitted by the use of those materials. In addition, marine derivate materials are a more economically and environmentally sustainable source than mammal counterparts [16–18].

Collagen/gelatin is widely referenced as building block of biomaterials with biomedical relevance for tissue engineering applications. Numerous studies in the literature report the good biocompatibility of marine collagen and it is referenced as key player for tissue regeneration [19–22]. More recently, studies performed by our group report the remarkable potential of this collagen for Tissue Engineering and Regenerative Medicine (TERM) [23–26], for example, in the fabrication of scaffolds for bone tissue regeneration [27] and also for cosmetical application [28].

The main goal in TERM is the efficient replacement and/or regeneration of damaged tissue or organs with a well-designed and appropriate construct. The stimulation and induction of a regenerative response by host cells and tissue can be developed appealing to the use of combinatorial biomaterials, cells and biochemical signals, but also by the biomaterial *per se* [29]. The success of this process is directly related to the response of the immune system to the material [30]. The activation of an inflammatory response can be the result of any tissue that is damaged. The chemokines and cytokines released by immune cells that are first recruited to the damaged site, regulate the further leukocyte response including migration, activation, and differentiation. The production of pro-inflammatory and anti-inflammatory cytokines are strictly related to the intensity and extension of the inflammatory response. Therefore, it can be used to characterize the type of inflammation and its severity [31,32].

Considering marine collagen as the biomaterial in question, to the best of your knowledge, there is a clear lack of information in the literature about its effect on the individual's immune system, existing only a few reports directly addressing the issue using marine collagen from squid [33], tilapia [31], jellyfish [19], and carp [32].

In this study, we use type I marine collagen and gelatin extracted from two different sources (codfish and blue shark skin) to assess the immunological response to these biomaterials. Both collagens and gelatin were previously extracted, and their physical and chemical characteristics had been already reported [28,34,35]. The immune response to the aforementioned materials was assessed *in vitro* using bone marrow-derived macrophages (BMDM). Also, an *in vivo* study was performed to evaluate the host response to an injectable form of the biomaterial.

2. Materials and methods

2.1. Preparation of collagen and gelatin solutions

Collagen from Codfish (*Gadus morhua*) (CC) was extracted and characterized in a previously study as described by Alves *et al.* 2017 [28]. Shark (*Prionace glauca*) collagen (SC) and gelatin (SG) were extracted and characterized as described by Diogo *et al.* [34] and Sousa *et al.* [35] respectively.. Freeze-dried collagen and gelatin films were previously sterilized by UV irradiation and then dissolved in 0.5 M of sterile acetic acid. Bovine collagen (BC) purchased from Sigma-Aldrich (#C2124, MO, USA) was used as mammal reference for *in vitro* studies.

2.2. Endotoxin assay

To measure the level of toxin contaminants, a solution of 1 mg/mL of each sample was prepared and analyzed for the presence of endotoxins. The quantification was performed using a Thermo Scientific Pierce LAL chromogenic endotoxin quantitation kit (#A39552, Thermo Fisher Scientific Inc., Rockford, IL, USA) following the manufacturer's instructions.

2.3. Animals

Eight- to 12-week-old C57BL/6 mice used for the *in vitro* studies were bred and housed at the Life and Health Sciences Research Institute (ICVS). For the *in vivo* studies, 50 healthy females C57BL/6 mice at 6 weeks of age (20-25 g) were purchased from Charles River Laboratories, Inc. (Barcelona, Spain) and randomly divided into 5 groups. All animal experiments were performed in strict accordance with recommendations of the European Union Directive 2010/63/EU and were previously approved by Portuguese National Authority for Animal Health–Direção Geral de Alimentação e Veterinária (DGAV). Mice were euthanized by cervical dislocation for BMDM isolation and by CO_2 inhalation for the *in vivo* studies with efforts to minimize suffering.

2.3.1. In vitro *studies*

2.3.1.1. Murine bone marrow-derived macrophages (BMDM). Murine bone-marrow progenitors were isolated from 8- to 12-week-old C57BL/6 mice. Briefly, the end of femur and tibias were cut-off, and the cells were flushed using complete Dulbecco's modified Eagle's medium (cDMEM) containing 10% heat-inactivated fetal bovine serum, 1% L-glutamine, 1% PenStrep, 1% HEPES and 1% of sodium pyruvate (all from Life Technologies). Cells were treated with erythrocyte lysis buffer (0.87% of NH_4Cl solution and 5% of PBS in water) for 2 minutes and then the suspension was centrifuged, and cell suspension concentration was assessed. For all in vitro studies, $5x10^5$ cells/mL were placed in 48 well plates and cultured at

37 °C under a 5% CO_2 atmosphere with the presence of 20 ng/mL of M-CSF (macrophage colony-stimulating factor). After 4 days, cell medium was supplemented with 20 ng/mL of M-CSF for 3 more days.

2.3.1.2. Collagen and gelatin titration. To find the best starting concentration of collagen/gelatin for cytokine production, a titration of those biomaterials was performed. Briefly, after BMDM differentiation, 25 mg/mL stock solution of CC, SC and SG, previously dissolved in 0.5 M of acetic acid and neutralized, were dissolved in cDMEM into a panel of different concentrations (2; 1; 0.5; 0.25; 0.125; 0.0625 and 0.03125 mg/mL). *E. coli* lipopolysaccharide (LPS) (0.1 mg/mL, Sigma-Aldrich, MO, USA) was used as positive control and neutralized acetic acid as negative control. These preparations were added to BMDM and incubated for 24 hours. Supernatant was collected from each condition and cytokine level measured.

2.3.1.3. BMDM stimulation with collagens and gelatin. To compare the immunogenicity between biomaterials and after titration, a concentration of 1mg/mL of material was settled for further experiences. After 7 days of differentiation with M-CSF, BMDM were stimulated with 1 mg/mL of CC, SC and SG diluted in cDMEM from a stock solution of 10 mg/mL. For BC, a solution of 1 mg/mL was prepared in cDMEM and neutralized before cells incubation. Cells were incubated for 1, 6 and 24 hours. The supernatants were collected for cytokine analysis and cells for qRT-PCR (quantitative Real-Time Polymerase Chain Reaction). During all time-points the morphology of cells were observed by optical microscopy.

2.3.2. In vivo *studies*

2.3.2.1. Intraperitoneal injection of mouse. To assess the *in vivo* immunological behavior of CC, SC and SG, C57BL/6 mice were intraperitoneally injected with these biomaterials as previously described by Miner *et al.* 1969 [36]. Briefly, 500 µL of CC (1 mg/mL); SC (1 mg/mL); SG (1 mg/mL); vehicle (as negative control) and casein (as positive control) was injected, and the mice were immunized during 6 and 24 hours and sacrificed at each time-point. To collect the peritoneal fluid (PF), mice were injected with 5 mL of DPBS to wash the cavity and the PF collected. The total number of cells was determined using a Neubauer chamber and cell recruitment was analyzed by flow cytometry.

2.4. Quantitative real-time PCR (qRT-PCR) analysis

Total mRNA was extracted using TRIzol™ reagent (Invitrogen, CA, EUA) following the manufacturerś instructions and cDNA was synthetized using Xpert cDNA Mastermix (GRiSP, Porto, Portugal) as described in manufacture's protocol. The expression levels of detected genes were quantified using KAPA SYBR® FAST qPCR Master Mix (2X) detection kit (Invitrogen, CA, EUA) by real time PCR (Bio-Rad CFX96 Real-Time System with C1000 Thermal Cycler). The relative mRNA levels of specific genes (Table 1) were normalized for ubiquitin (*Ubq*) and assessed by the $2\Delta^{Ct}$ method.

2.5. Enzyme-linked immunosorbent assay (ELISA)

The levels of cytokines present on cell-free supernatant of stimulated BMDM were evaluated with the Invitrogen kit according to manufacturer's instructions: mouse TNF-α (cat # 88-7324-88); mouse IL-10 (cat # 88-7105-88); mouse IL-1β (cat # 88-7013-88), and mouse IL-6 (cat # 88-7064-88).

2.6. Flow cytometry analysis

Cells collected from peritoneal cavity were characterized using flow cytometry. The cells were washed with FACS buffer (3% FBS and 0.01% sodium azide in PBS) and immunostained with Pe/Cy7-conjugated anti-CD11b (clone:M1/70; Cat:101216), BV605-conjugated anti-CD11c (clone:N418; Cat:117334), PerCP/Cy5.5-conjugated anti-Ly6C (clone:HK1.4; Cat:128012) and BV785-conjugated anti-Ly6G (clone:1A8; Cat:127645) antibodies (all from BioLegend, CA, USA). After washing with FACS buffer, cells were fixed with 2% paraformaldehyde. Samples were acquired on a LSR II flow cytometer with FACSDiva software (BD Bioscience). Data were analyzed using FlowJo software (TreeStar).

Table 1
Primers sequence used for amplification in real-time PCR.

Gene	Sequence (5′-3′)	
	Forward	Reverse
Tnf	GCCACCACGCTCTTCTGTCT	TGAGGGTCTGGGCCATAGAAC
Il6	CTCATTCTGCTCTGGAGCCC	GTGACTCCAGCTTATCTCTTGGT
Il1b	GGGCTGCTTCCAAACCTTTG	AAGACACAGGTAGCTGCCAC
Il10	ATTTGAATTCCCTGGGTGAGAAG	CACAGGGGAGAAATCGATGACA
Nos2	ACAGGGAGAAAGCGCAAA	AGTGAAATCCGATGTGGCCT
Arg1	TTTTAGGGTTACGGCCGGTG	CCTCGAGGCTGTCCTTTTGA
Ubq	GGCTATTAATTATTCGGTCTGCAT	GCAAGTGGCTAGAGTGCAGAGTAA

Tnf (Tumor necrosis factor alpha); *Il6* (Interleukin-6); *Il1b* (Interleukin-1 beta); *Il10* (Interleukin-10); *Nos2* (nitric oxide synthase 2); *Arg1* (Arginase 1); *Ubq* (Ubiquitin).

2.7. Statistics

Statistical analysis was performed using SPSS software (IBM SPSS Statistics version 25, Armonk, NY, USA. Shapiro-Wilk and Levene tests were applied to verify the data normality and homogeneity of variances, respectively. In case of experiments conducted over time, the data fail to pass these tests as such, a Kruskal-Wallis test followed by Fisher's LSD test was performed. In case of *in vivo* experiments, unpaired *t*-test (Mann Whitney-U-test) for non-normal distributed variables was performed. Statistical significance was defined as $p < 0.05$. Data were expressed as means ± standard deviation of experiments with at least two independent experiments.

3. Results and discussion

3.1. Presence of endotoxins in fish skin collagen and gelatin

Collagen and gelatin products are considered as Generally Recognized As Safe (GRAS), when used in accordance with Good Manufacturing Practices (GMP), by the US Food and Drug Administration (US FDA) and compliant with the US, European, and Japanese Pharmacopeia [37–40]. However, even when produced under medical grade conditions, the presence of contaminants, such as endotoxins, could compromise the safety of the material. Endotoxins are a component of the outer cell membrane of gram-negative bacteria and when in contact with the blood stream may cause an immunological reaction that can go from an acute inflammation to a systemic sepsis [41]. To have an accurate understanding of the inflammatory nature of our biomaterials, the endotoxin levels were quantified by LAL (*Limulus* Amebocyte Lysate) method and the obtained values are depicted in Table 2. Although the determined endotoxin levels were above the FDA guidelines (below 0.5 EU/mL or 20 EU/device for medical devices in general and below 0.06 EU/mL or 2.15 EU/device for devices in contact with the cerebrospinal fluid [41]), the materials were not extracted under medical grade conditions and thus, the presented values could have been influenced by the extraction conditions, from which we decided to proceed. Endotoxin levels may be reduced using additional procedures, as ultrafiltration membrane with an appropriate molecular weight cut-off [42] or a special buffer and resin [43], but besides the associated additional costs, there may be also an

Table 2
Levels of endotoxin present in the marine biopolymers, assessed via LAL test.

Sample	[Endotoxin] EU/mL		[Endotoxin] ng/mL
	M*	SD**	
SG	2.33	0.020	0.47
SC	0.59	0.005	0.12
CC	3.63	0.026	0.73

* Mean (M);
** Standard Deviation (SD)

impact on the properties of the molecules, namely at structural level. The endotoxin concentration was also converted from EU/mL to ng/mL for a better correlation between the concentration of endotoxins in our materials and the LPS concentration used during further studies.

3.2. Effect of fish collagen and gelatin in cytokine release by BMDM

When an exogenous material is implanted into a living tissue, a natural and inevitable response is initiated by the host [44]. This immune response to the biomaterial not only depends on the amount and quality of the material but also its intrinsic properties [45–49]. For this reason, we studied the dose effect of the different materials on cytokine expressed by immune cells. BMDM are *in vitro* differentiated cells derived from bone marrow stem cells and belong to the innate immune system, providing a first line defense against foreign bodies. BMDM offers a rapid response to the surrounding environment by presenting a fast polarization (surface molecular marker presentation, gene expression/repression and chemokines/cytokine expression) given a quick resolution to either pro-inflammatory or pro-resolving stimuli [50]. Also, being primary cells differentiated from stem cells, are naïve regardless the health issues of the donor and get closer to the biological features of an *in vivo* model [51]. The materials were incubated for 24 hours with BMDM, the results of the evaluated cytokine profile by ELISA assay are depicted in Fig. 1. A panel of four cytokines, based on their role in the establishment and regulation of immune responses was chosen, including TNF-α, IL-6 and IL-1β, and IL-10. Of notice, IL-1β was not detected (data not showed), not even after LPS stimulation. This event may be justified by the absence of the additional stimulus involving the activation of the inflammasome and caspase-1 that is required for proteolytic cleavage and secretion of mature IL-1β [52–54].

Considering that some levels of endotoxins were detected in our materials (Table 1), LPS was chosen as control and inflammatory reference in a range of 200 ng/mL to 3.13 ng/mL, where the maximum concentration is considered above the optimal to be used [55]. LPS is one of the major outer membrane components of Gram-negative bacteria and is responsible for triggering several inflammatory cytokines that can lead to an acute inflammation state (septic shock). It is also a potent activator of monocytes and macrophages by inducing the production of several cytokines and chemokines such TNF-α, IL1β, IL6 and nitric oxide [56–58]. For all cytokines analyzed, a clear pattern of decreasing LPS dose-response was observed. At the lowest concentration (3.13 ng/mL), no production of TNF-α, IL-6 or IL-10 was detected and because of that we felt no need of additional titration. For further experiments, we choose a LPS reference concentration that is commonly used in biological assays, 100 ng/mL, that corresponds to a concentration about 130 times higher when compared with CC, the material studied with higher endotoxin level. This allows us to realize that our samples presented lower endotoxins levels and thus, the results observed are only related with the nature of material and not with a possible endotoxin effect.

The concentrations tested in our materials ranged from 2 mg/mL to 0.0313 mg/mL. In general, it was observed the same descendent dose-response pattern as observed for LPS. Nevertheless, for the titration of SG for TNF-α and IL-6, and of CC for IL-6 there is a non-monotonous decrease. It would be natural to think that this phenomenon would be due to the fact that for higher concentrations, the collagenous material could form a fiber network and start some kind of polymerization that would give origin to insoluble material and therefore, to a less amount of soluble material available for macrophage internalization. Nevertheless, this aspect does not occur in our samples since collagens from cold water fishes, as are codfish and shark, present low gelling temperatures [28,35], which means that temperatures below 12 °C are needed to initiate fiber self-assembly, contrasting with some mammal collagenous materials in which this type of phenomenon takes place at 37 °C. We think that this feature may be due to a common effect of down-regulation where, in some cases, a high dosage of a compound leads to overload and produce an inverse response. The behavior might be similar to the one observed with T cells stimulated with anti-CD3, where a high dose induces down regulation of the receptor and lower doses lead to a low stimulation [59,60]. A closer look in the results showed that in general, for lower doses (0.125 mg/mL – 0.0313 mg/mL) of biomaterial, the cytokine release is very low or even non-detectable and could not be considered as reference concentration to be use in further tests. In a previous study of our group where the effect of CC in cell viability and metabolism was assessed [24], it was demonstrated that for concentration above 1 mg/mL, cell adhesion and metabolism was compromised. Taking this into account with other similar reports [33] and putting together all the analyses, it was decided to focus on the dose of 1 mg/mL to compare the effect of the different materials and use it as reference dose to perform further studies.

Looking at Fig. 1 and for the treatment with 1 mg/mL, it is possible to observe that we had distinct responses to the different biomaterials. As expected, when a foreign material contacts with this type of cells, a cytokine profile is immediately released to help to identify the hazardous levels and reestablish the tissue homeostasis [61]. For instance, SC demonstrated to be the less immunogenic biomaterial, with very low production of TNF-α and IL-6 when compared with the other biopolymers, especially SG ($p < 0.005$). Oppositely, SG exhibited higher levels of pro-inflammatory cytokines, particularly IL-6, considering this the biomaterial capable of triggering a superior inflammatory reaction. In relation to CC, this was the biomaterial that presented an intermediate cytokine release. However, a common point between all the materials is the release of anti-inflammatory cytokine IL-10. Particularly, SG that showed higher levels of pro-inflammatory cytokines (TNF-α and IL-6), also revealed considerable levels of IL-10 ($p < 0.05$). Usually, IL-10 acts as suppressor of many effector cells such macrophages, monocytes, dendritic, neutrophils, eosinophils and mast cells, through inhibition of pro-inflammatory cytokines and chemokines, cell maturation and activation [62]. This cytokine, a potent anti-inflammatory cytokine, is produced by most of the cells of the immune system including M2-like macrophages, is well known by its critical role in the regulation of immunity response and mediation of the inflammatory state [63–65] as well as in scar formation, and in the regeneration of both embryonic and adult cells [66,67]. This could be an indicator that our materials are able to stimulate the differentiation of these type of macrophage and thus, when implanted as biomaterial in the context of tissue engineering, they may have a positive effect on stimulating the remodeling of the tissue.

We also have performed these analyzes on a commercial bovine collagen (BC) to act as a mammal collagenous reference (Supplementary Fig. 1). What we observed was that no expression or only a residual expression was detectable for TNF-α and IL-6, both pro-

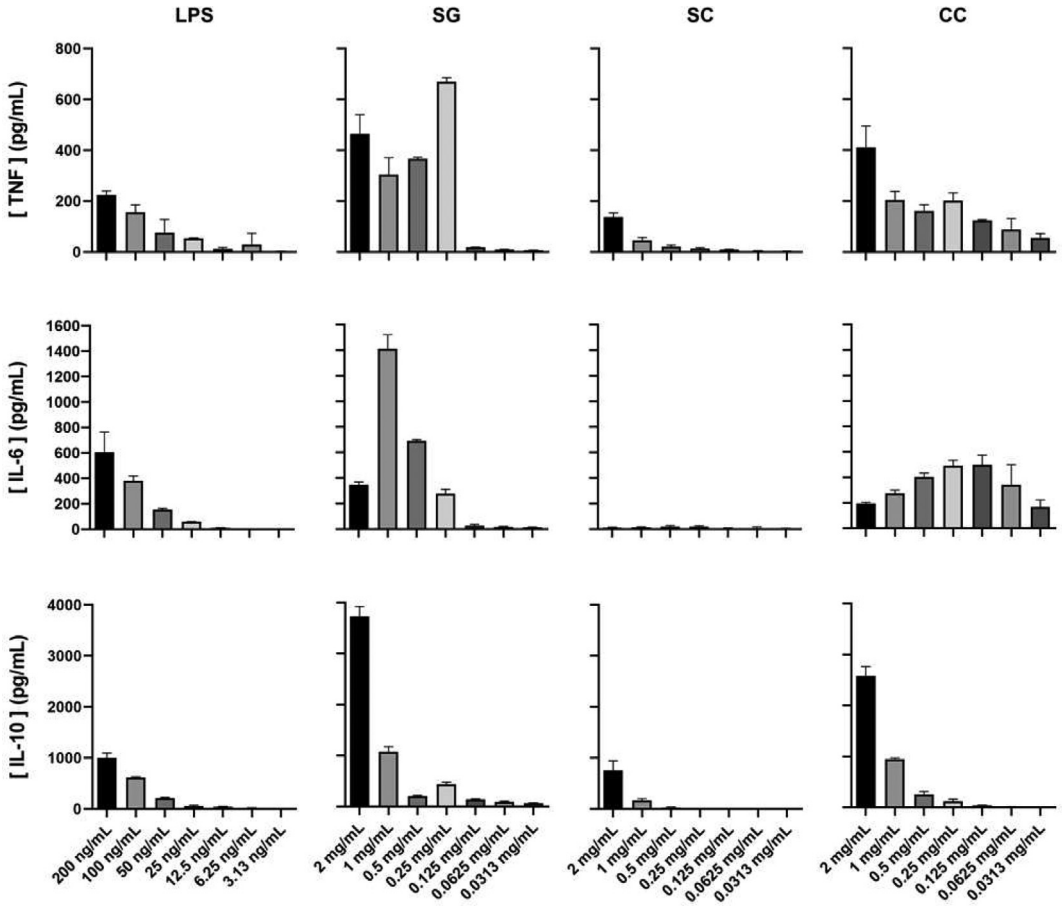

Fig. 1. Quantification of cytokine levels during collagen and gelatin stimulation. Quantification of TNF-α; IL-6 and IL-10 levels in BMDM supernatants after 24 hours of stimulation with different concentrations of SG, SC and CC by ELISA assay. LPS was used as control of inflammation.

inflammatory cytokines, whereas expression was observed for LPS condition (positive control). Similarly, only a residual expression was obtained for IL-10 cytokine when cells went exposed to BC. These results were, in some way, already expected since this is a commercial sample that follows all the GMP manufacturing guidelines and is a widely used biomaterial tool to enhance cellular support or even for the production of 3D scaffolds for tissue applications [68–70]. These data suggest that bovine collagen is neutral for the macrophage population and does not incite any type of cell polarization.

3.3. Effect of fish collagen and gelatin in BMDM gene expression relevant markers

Besides the protein quantification, it is also important to assess cytokines gene expression to understand and correlate with earlier events that could occur. Therefore, the effect of fish collagen and gelatin stimulation at gene expression level in BMDM was evaluated after 1, 6 and 24 hours of treatment for *Tnf, Il6 and Il1b, Il10* (Fig. 2). Considering pro-inflammatory cytokines, when compared with control of cells, it was possible to observe an increase of relative mRNA expression at 1 and 6 hours for *Tnf* in all biomaterials with an evident decrease of expression at 24 hours of incubation. For *Il6*, an increase at 6 hours of stimulation for SG and CC was observed. This expression, as in *Tnf*, return to basal levels after 24 hours. The levels of *Il1b* on BMDM increased when exposed to SG and CC at 6 hours, being extended during the 24 hours. Looking for *Il10*, we could see an increase of mRNA levels at an early time, 1 hour, with a clear time-related decrease for all biomaterials. SC was the biomaterial that presented the major increase but with a drop at 6 hours, and SG and CC revealed a smooth decline during time. This behavior is in line with what was also observed by the protein expression results. Also, the gene expression levels of enzymes inducible nitric oxide synthase (*Nos2*) and arginase 1 (*Arg1*) related with the regulation of differentiation and function of immune cells, namely macrophages, were analyzed. It is evident that only a basal expression of *Nos2* was detected in all time-points. On the other hand, an increase of *Arg1* expression was detected at 24 hours of stimulation, being more emphasized for SG and CC.

As in the protein expression, also the effect of BC in gene expression of these cytokines and enzymes was assessed (supplementary Fig. 2). Again, only a residual expression was detected for all genes analyzed, with the exception of *Tnf* where expression was detected at 1 h of stimulation with a decrease pattern over time (6 h and 24 h). In what regard the expression of the enzymes, *Nos2* and *Arg1* was also considered absent being limited to a basal cellular level in all timepoints. These results are in accordance with what was observed for the cytokine release.

Macrophages are known for its heterogeneity offering versatility and plasticity when exposure to different microenvironments. As they sense the surround environment, they have the capacity to differentiate in different lineages and depending on that, act by different ways. In a simplistic view, M1-like macrophages are linked to the production of pro-inflammatory cytokines (IL-1, IL-6 and TNF-α), to kill pathogen-infected cells, to express inducible NO synthase, and to initiate an acute immune response. On the other hand, M2-like macrophages are related with wound healing and tissue repair being responsible for the production

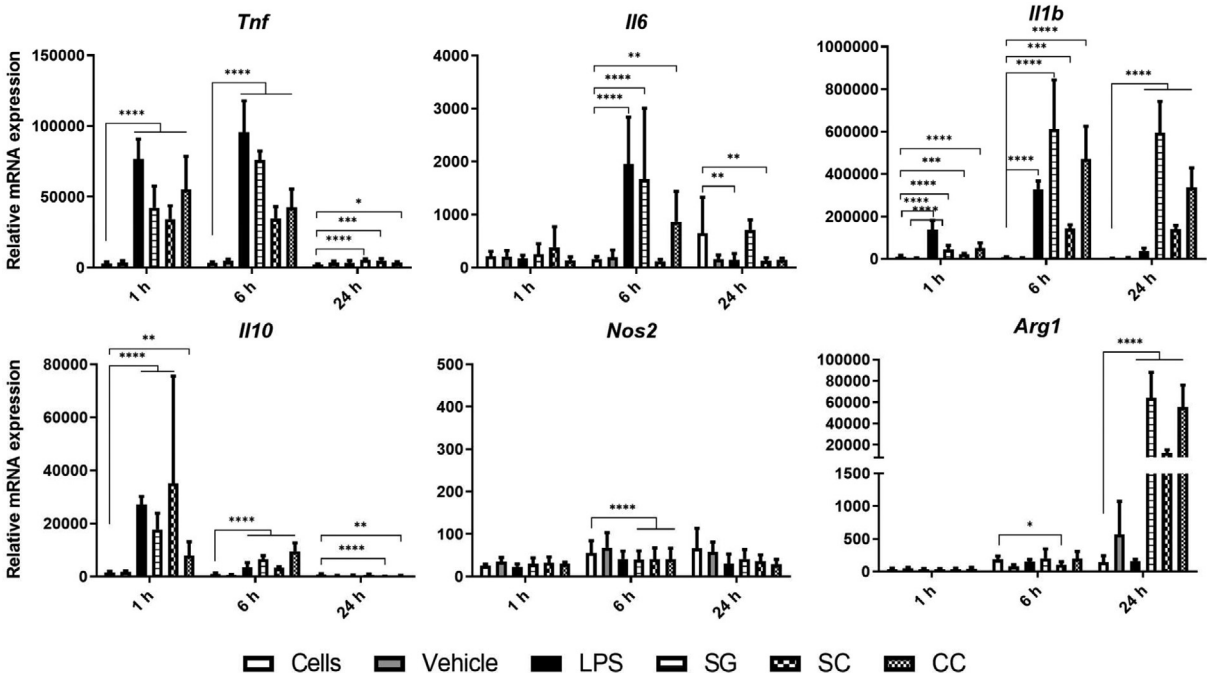

Fig. 2. Gene expression of key cytokines and enzymes. Relative mRNA expression levels of *Tnf; Il6; Il1b; Il10; Nos2* and *Arg1* after 1, 6, and 24 hours of stimulation with 1 mg/mL of SG, SC and CC, and 100 ng/mL of LPS. Quantification of mRNA expression was calculated after normalizing signals against ubiquitin. Statistical significance was defined as $p < 0.05$ in comparation to Cells. $^{*}p \leq 0.05$, $^{**}p \leq 0.005$, $^{***}p \leq 0.0005$, $^{****}p \leq 0.00005$.

of IL-1R antagonist, IL-10, and arginase [71–73]. As reported by Katakura *et al.* (2004) [74], IL-10 acts as immunosuppressor associated with alternatively activated macrophages (also known as M2-like macrophages), being able to block the generation of classically activated macrophages (also known as M1-like macrophages) then inhibiting the progression of the inflammatory to an acute state. Also, Chao Liu and Jiao Sun [75] reported that fish collagen hydrolysates have the potential to polarize human macrophages into M2-like phenotype through inhibition of the expression of M1 macrophage markers (IL1β and TNF-α) and enhancing the expression of M2 macrophage marker (Arg1 and IL-10). Other study from the same authors suggested that bone marrow-derived mesenchymal stem cells treated with fish collagen hydrolysates are able to induce some immunoregulatory factors such TNF-α - induced gene/protein 6 (TSG-6, associated with anti-inflammatory effect) and that, when co-cultured with macrophages, exert a immunomodulatory effect that lead to the inhibition of TNF-α and IL-12 and promotion of expression and secretion of IL-10 and CCL22 [76].

The evidences observed for gene expression of IL-10, for all the marine materials studied, at an earlier stage and the higher quantification of this cytokine (as discussed in the previous section), associated with low gene expression of NOS2 and high gene expression of Arginase, observed in our study, seem to indicate that our biomaterials promote the differentiation into a M2-like macrophage population. Also, when compared with bovine collagen, our marine materials seem to be promising and have a higher potential as immunomodulators of the surround environment given that bovine collagen does not stimulate either M1 or M2-like macrophage phenotype and acts as a neutral player, from which the marine derived collagenous compounds could be seen as alternative material.

3.4. Effect of SG, SC and CC injection over host immune system

To understand the effect of collagen and gelatin material *in vivo*, each of the materials were injected in the peritoneal cavity

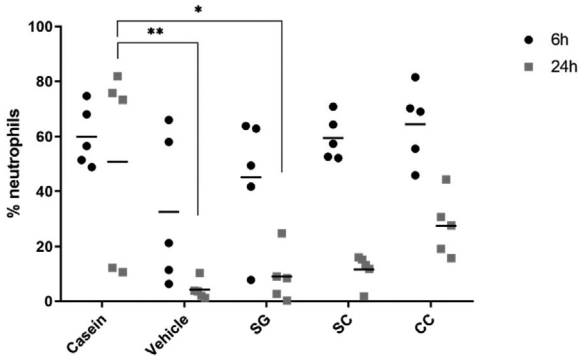

Fig. 3. Quantitative analysis of neutrophil cell population in peritoneal cavity. One mg/mL of biomaterials were injected in peritoneal cavity and analyzed by flow cytometry after 6 and 24 hours. Statistical significance was defined as $p < 0.05$ in comparation to casein. $^{*}p \leq 0.05$ and $^{**}p \leq 0.005$. The gating strategy employed for flow cytometry experiments is shown in Supplementary Figure 1.

of C57BL/6 mice together with a casein and a vehicle group. We chose to use casein as an inflammation control group since we are well aware of the immune dynamics created after its injection [77]. The injection of SG, SC and CC was performed without any complications. All animals responded properly to the procedure and thrived well without any signs of further problems. To identify immune cell subsets, present in the peritoneal cavity of animals, we used high-dimensional flow cytometry (i.e., FACS). Data from peritoneal lavage cells were collected from treated mice exposed for 6 hours and 24 hours and gated for single live cells from leukocyte population (Supplementary Fig. 3). During the first 6 hours, it was possible to observe a positive and strong recruitment of pan myeloid derived neutrophil population ($CD11b^{+}$ $Ly6G^{+}$) in all biomaterials (Fig. 3). After 24 hours, the neutrophil population decreased significantly being comparable to vehicle condition, particularly for shark- derived materials SC and SG. Neutrophils are commonly the first line of defense of the innate immune system, being

the first cells to come into contact with biomaterials, also playing an important role in the resolution of inflammation and tissue regeneration [78]. The importance of comparing different phlogistic agents is related to the ability of these agents to induce different cellular dynamics, regardless of the cytokine expression profile. Their pathogen killing system passes by using multiple tools that allows to interact, influence, recruit and secrete signals for the surround immune and humoral cells [79,80]. This type of immune cells present a short-life time of only a few hours in circulation (about 12.5 hours for mouse cells [81]) but can easily extend up to 3 days if the inflammatory state shows off to be severe and chronic through a continuous recruitment into the tissue [82–84]. It is clear that our materials do not elicit a strong or continuous recruitment of neutrophils as shown by the decrease of these cells after 24 hours of exposure to the materials. The neutrophils recruited at earlier stages (6 hours) experienced a normal process of spontaneous apoptosis and were removed by neighboring macrophages and dendritic cells via phagocytosis, leading space to the resolution of the inflammatory process [85,86].

Additionally, neutrophils can show some plasticity since they can alter its surface markers according to the inflammatory environment. Different subsets of pro-inflammatory nature (IL-12^{+}), CC-chemokine ligand 3 (CCL3) and anti-inflammatory (IL-10^{+}, CCL2) can be found in inflammatory milieu [80,87]. The existence of these neutrophil populations can, somehow, shape the course of the immunological response by inducing the recruitment of M1-like or M2-like macrophages, respectively [88]. It is important to highlight the relevance of M2/M1 ratio to promote a healthy immune response. A permanent balance between M1-like macrophages associated with chronic inflammation and M2-like macrophages linked with regeneration is crucial, and the switch between both is tightly influenced by neutrophil [78]. Although, commonly known as contributors of establishing the chronic inflammation and contribute to tissue damage, neutrophil can also have a role on tissue repair and regeneration and be involved in processes related with inflammation resolution by changing their phenotype [89]. Apoptotic neutrophils are responsible for macrophage attraction which in turn, induces a pro-resolving cascade, including the release of repair cytokines such as TGF-β, IL-10, and VEGF, leading to macrophages reprogramming to an anti-inflammatory phenotype [90]. The IL-10 expression is associated with the phase of inflammation resolution, not only because is one of the key cytokine for M2-like macrophage phenotype recruitment but also with neutrophil repair profile induced by this cytokine [89].

Although additional studies needed to be performed, namely the screening of surface markers of these two types of neutrophils and the chemokine and cytokine profile released by them, we could see a relation between the expression of IL-10, induced by our materials, showed on Figs. 1 and **2**, and the transient role observed in neutrophil population. Besides IL-10, the strong gene expression of *Arg1* over *Nos2* (Fig. 2), induced by our marine materials, is a marker of M2-like macrophage phenotype prevalence and an indicator that a pro- resolving cascade is being started. Surely, the recruited neutrophil population will play an important role in this relationship.

4. Conclusion

The immunological effects of mammal collagens and gelatin are object of studies for several years. Recently, the growing interest on marine-derived collagen and gelatin has brought to the light the concerns about the effect of the use of those biopolymers as raw material to produce the biomaterial or its use in cosmetic formulation, in hosts system. Most studies that present marine origin collagen or gelatin as biomaterial for biomedical or wellbeing applications, only refers to its compatibility with cells. Few studies have been performed directly addressing the immunological impact whether they are using it as raw material or already processed into a scaffold. In this study, we showed the impact of collagen and gelatin solutions extracted from skins of blue shark, and codfish over bone marrow-derived macrophages and in peritoneal cavity of mice.

It is possible to state that for the *in vitro* experiments, shark collagen (SC) is the material that induced lower expression of pro-inflammatory cytokines. Shark gelatin (SG) was the material that presented higher pro-inflammatory cytokine expression, however, along with cod collagen (CC), are the materials that presented a significant expression of IL-10 and Arginase, both key players on M2-like macrophage phenotype related with anti-inflammation resolution. The *in vivo* study of neutrophil recruitment demonstrated that none of our materials provoked an exacerbated response and no acute inflammation seemed to be found at 24 hours of injection.

Although more profound studies regarding each intervenient of innate immune response as well as long-term exposure of materials should be performed, we can see a relation between the biomaterials and the expression of key players in the differentiation and induction of anti-inflammatory regulators. Above all, our materials exhibited a satisfactory immune performance with the promising outcomes in its use as immune modulators and anti-inflammatory inducible materials.

Data availability

The raw/processed data required to reproduce these findings cannot be shared at this time due to technical or time limitations.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

A.L. Alves: Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft, Writing – review & editing. **J. Costa-Gouveia:** Methodology, Formal analysis, Investigation, Writing – review & editing. **J. Vieira de Castro:** Investigation, Writing – review & editing. **C.G. Sotelo:** Resources, Investigation, Writing – review & editing. **J.A. Vázquez:** Resources, Investigation, Writing – review & editing. **R.I. Pérez-Martín:** Conceptualization, Investigation, Writing – review & editing, Supervision, Funding acquisition. **E. Torrado:** Validation, Investigation, Writing – review & editing. **N. Neves:** Investigation, Writing – review & editing. **R.L. Reis:** Resources, Writing – review & editing, Project administration, Funding acquisition. **A.G. Castro:** Conceptualization, Methodology, Validation, Resources, Writing – review & editing, Supervision. **T.H. Silva:** Conceptualization, Methodology, Validation, Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.actbio.2022.01.009.

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