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Macrophage polarization following chitosan implantation

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ABSTRACT

Macrophages are a key cell in the host response to implants and can be polarized into different phenotypes capable of inducing both detrimental and beneficial outcomes in tissue repair and remodeling, being important in tissue engineering and regenerative medicine. The objective of this study was to evaluate the macrophage response to 3D porous chitosan (Ch) scaffolds with different degrees of acetylation (DA, 5% and 15%). The M1/M2 phenotypic polarization profile of macrophages was investigated *in vivo* using a rodent air-pouch model. Our results show that the DA affects the macrophage response. Ch scaffolds with DA 5% induced the adhesion of lower numbers of inflammatory cells, being the M2 the predominant phenotypic profile among the adherent macrophages. In the inflammatory exudates F4/80$^+$/CD206$^+$ cells (M2 macrophages) appeared in higher numbers then F4/80$^+$/CCR7$^+$ cells (M1 macrophages), in addition, lower levels of pro-inflammatory cytokines together with higher levels of anti-inflammatory cytokines were found. Ch scaffolds with DA 15% showed opposite results, since M1 were the predominant macrophages both adherent to the scaffold and in the exudates, together with high levels of pro-inflammatory cytokines. In conclusion, Ch scaffolds with DA 5% induced a benign M2 anti-inflammatory macrophage response, whereas Ch scaffolds with DA 15% caused a macrophage M1 pro-inflammatory response.

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1. Introduction

Implantation of a biomaterial causes injury that will lead to the onset of inflammation which is a prerequisite for efficient tissue repair; nonetheless, successful repair after tissue injury requires resolution of the inflammatory response. Persistence of an inflammatory stimulus results in chronic inflammation that is a hallmark of the non-healing wound. Therefore, successful biomaterial integration requires the coordinate expression of both inflammation, at first, and resolution of inflammation, latter on [1].

Leukocytes are central players in directing host inflammatory and immune processes; thus, their response to biomaterials is important in the understanding of material–host interactions. Monocytes are recruited to the implant site, undergo maturation into macrophages, and persist at implant surfaces. Macrophages are the dominant infiltrating cells that respond rapidly to biomaterial implantation in soft and hard tissues. Macrophages may play a bimodal role: by the release of pro-inflammatory cytokines, participate in the initiation of an acute inflammatory response, and by the release of anti-inflammatory cytokines, pro-angiogenic factors and matrix metalloproteases (MMPs), participate in the biodegradation of bioresorbable implants, playing an important role in tissue regeneration and healing. By phagocytosis, macrophages clear the way for tissue ingrowths, secrete a spectrum of cytokines and growth factors to regulate cell recruitment, proliferation and differentiation, leading to effective tissue regeneration and angiogenesis. Thus, macrophages have been suggested to be the cells that orchestrate both the inflammatory and the repair phase of tissues around implants [2,3].

Macrophages display remarkable plasticity and can change their physiology in response to environmental cues, giving rise to different cell populations with distinct functions. This is reflected in the division of macrophages into two major phenotypes (M1 and M2). The pro-inflammatory, cytotoxic macrophage phenotype, labeled as M1 (classically activated), promotes pathogen killing and

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is associated with classic signs of active inflammation, particularly with chronic inflammation. The anti-inflammatory macrophage phenotype, labeled as M2 (alternatively activated), promotes immunoregulation tissue repair and constructive tissue remodeling. M1 and M2 macrophages can be identified and distinguished according to their cell surface markers and their cytokine and gene expression profiles [4–6].

Chitosan, a biodegradable polysaccharide obtained by N-deacetylation of chitin, is a natural polymer that is currently under investigation for several biomedical applications, namely as scaffolds and as drug and gene delivery system for regenerative medicine [7,8]. Previous results of our team have demonstrated that the degree of acetylation of Ch affects the behavior of osteoblasts [9] and also modulates the *in vivo* biological response, namely inflammatory cell recruitment and fibrous capsule formation around implants [10].

The ability of the host innate immune system to resolve a polarized macrophage response (M1 or M2) resulting from an implant is of key importance to determine the downstream functional success of implanted biomaterials. However, few studies have evaluated macrophage polarization specifically with respect to biomaterials implantation [11]. The effects of macrophage phenotype upon tissue remodeling outcome following implantation of a biomaterial are largely unknown. However, the recognition of the predominant phenotypic profile may provide a tool by which a constructive and functional tissue remodeling outcome can be predicted and/or promoted [12].

The objective of the present study was to determine the effect of implantation of 3D chitosan scaffolds on the phenotypic profile of the macrophages participating in the host response, and also the relationship between the degree of acetylation of chitosan and macrophage polarization.

2. Materials and methods

2.1. Chitosan (Ch) purification and characterization

Squid pen Ch (ref. 114, Batch No. S4; DA ~2%) was supplied by Mahtani Chitosan Pvt. Ltd. and subsequently purified by filtration of chitosan acidic solution and subsequent alkali precipitation. The purified Ch was characterized in terms of DA and average molecular weight by Fourier transform infrared spectroscopy (FT-IR) and size exclusion chromatography, respectively, as previously described [9]. A DA of 5.02 ± 0.66 ($n = 6$), with weight-average molecular weight (M_w) $8.9 \pm 1.0 \times 10^5$ and polydispersity index (PDI) 1.2 ± 0.0 were found. Chitosan with DA 15% was prepared by N-acetylation of the former, according to Vachoud L et al. [13], in a water/acetic acid/1,2-propanediol solution, using acetic anhydride as reactive. Subsequent analysis revealed a DA of 15.71 ± 0.52 ($n = 6$), M_w $8.3 \pm 0.9 \times 10^5$ and a PDI of 1.3 ± 0.1. Chitosan endotoxin levels were measured in water extracts, using the Limulus Amebocyte Lysate (QCL-1000® test, Cambrex), for the chromogenic quantitation of Gram-negative bacterial endotoxin. Briefly, Ch extracts were prepared using 40 mL endotoxin-free water/g of Ch, incubating Ch suspensions for 24 h at 50 °C under continuous shaking (250 rpm), as described elsewhere [14]. The resultant extracts were filtered through a 0.45 μm syringe filter, and used for endotoxin quantification. The two polymers revealed endotoxin levels below 0.1 EU/mL, respecting the US Department of Health and Human Services guidelines for implantable devices.

2.2. Preparation and characterization of Ch 3D scaffolds

The 3D porous scaffolds were prepared from degassed 2% *w*/*v* Ch solutions in 0.2 M acetic acid via thermally induced phase separation (−20 °C) and subsequent sublimation of the ice crystals. Following lyophilization (−85 °C; 0.2 mbar), the resultant scaffolds were cut in discs with 8.5 mm in diameter and 2 mm thickness. Scaffolds microstructure was analyzed by scanning electron microscopy (SEM) in transversal and longitudinal cross-sections of the lyophilized scaffolds. In accordance to previous results Ch scaffolds revealed a highly porous and homogeneous microstructure with interconnected pores with diameters in the range of 100 μm, for both the DAs used [10].

2.3. Mouse animal model

The procedures involved in the animal model were submitted and evaluated by the in-house ethics committee and also by the Portuguese official authority on animal welfare and experimentation (DGV), and were approved before the experiments were performed.

For each experimental group, 6 male BALB/c mice (Charles River, Spain) were used at 7 weeks of age. Air pouches were generated according to the method of Sedwick et al. [15] as adapted by Castro et al. [16]. Anaesthetized by intramuscular injection of ketamine (Ketalar, Parke-Davis Co., Spain; 4.0–8.0 mg/kg of weight) and xilazine (Rompum, Bayer Co., Portugal; 0.8–1.6 mg/kg) mice were injected subcutaneously in the dorsal area with 5 mL of sterile air that caused the formation of an air pouch. A reinforcement of the air pouch was performed 5 days latter through a second subcutaneous injection of 3 mL of sterile air. A single scaffold was implanted in each animal.

2.4. Implantation of the chitosan 3D scaffolds

One day after the second subcutaneous injection, the mice were anaesthetized by intramuscular injection of ketamine (Ketalar, Parke-Davis Co., Spain; 4.0–8.0 mg/kg of weight) and xilazine (Rompum, Bayer Co., Portugal; 0.8–1.6 mg/kg) and the skin covering the air pouch area was shaved and cleaned with betadine. A surgical incision was made, the materials were placed inside the air pouch, and the incision was sutured. Three different experimental groups were used: One group with Ch scaffolds with a DA 5%, another group with Ch scaffolds with DA 15% and sham-operated animals (animals submitted to the same technique with no scaffold implanted). For each experimental group 6 animals were used.

2.5. Inflammatory exudates

The exudates were recovered from the mouse air-pouches 1 and 4 days after the implantation. The mice were anaesthetized and sacrificed. Harvesting of inflammatory exudates was done by washing the air pouch cavities with 2 mL of phosphate buffered saline (PBS, Merck) followed by recovery of the lavage fluid.

2.6. Explants

Explantation occurred immediately after the recovery of the inflammatory exudates, 1 and 4 days after implantation. The sutures were cut, and the wound edges separated; the scaffolds were then carefully removed from the pouches and fixed with 4% paraformaldehyde (PFA, Merck) in PBS for 1 h, for confocal microscopy.

2.7. Confocal microscopy

Chitosan scaffolds were rinsed in PBS three times in order to remove any traces of PFA. Non-specific binging sites were blocked with 1% BSA (bovine serum albumin, Gibco), 10% FBS (fetal bovine serum, Gibco) and 5% NGS (normal goat serum, Invitrogen) and permeabilized with 0.1% Triton-X (Sigma) in PBS for 1 h at room temperature. Samples were incubated overnight at 4 °C with rat monoclonal antibody α-F4/80 (1:200), mouse monoclonal antibody α-Mannose Receptor CD206 (1:100) and rabbit monoclonal antibody α-CCR7 (1:350), all from Abcam. After washing three times with PBS, the scaffolds were incubated for 1 h at room temperature with secondary antibodies Alexa Fluor® 488 rabbit α-rat (1:1000), Alexa Fluor® 568 goat α-mouse (1:1000) and Alexa Fluor® 647 goat α-rabbit (1:1000), all from Invitrogen. For F-actin staining, scaffolds were prepared as above and stained with Alexa Fluor® 488 Phalloidin (1:100, Invitrogen). All scaffolds were mounted with Vectashield® with DAPI (Vector). Images were obtained with a laser scanning confocal microscope Leica TCS SP5 II (Leica Microsystems, Germany). An average of 5 horizontal image sections with a z-step of 50 μm were obtained per site and a total of 5 sites per scaffold were visualized. In order to render the 3D image into a 2D projection, the Maximum Intensity Projection was performed. All images were processed with Fiji software. Confocal images of M1 and M2 macrophage colonization of the implanted scaffolds were further scored into three categories: Present (+); Frequent (++); Abundant (+++).

2.8. Flow cytometry

The exudates were filtered through 40 μm nylon mesh (BD Biosciences) to remove cell clumps and spun at 1200 rpm for 5 min at 4 °C. Supernatants were removed and cell pellets re-suspended in 1 mL staining buffer (PBS/0.5% BSA/0.1% azide). Single-cell suspensions were pre-incubated with Fc-receptor-blocking antibodies (Miltenyi Biotec) for 10 min at 4 °C. Labeling was performed in a final volume of 50 μl with the indicated fluorescently conjugated antibodies for 30 min at 4 °C in the dark. Cells were then washed three times with staining buffer and transferred to FACS tubes for flow cytometry analysis. The following antibodies were used in this study: phycoerythrin (PE)-labeled anti-mouse F4/80 (clone 521204, 4:50 μl), fluorescein isothiocyanate (FITC)-labeled anti-mouse CD3 (clone 17A2, 3:50 μl), allophycocyanin (APC)-labeled anti-mouse CCR7 (clone 4B12 7:50 μl), Alexa Fluor®488 labeled anti-mouse MMR/CD206 (3:50 μl), all from R&D Systems and APC-labeled anti-mouse CD11b (Clone M1/70.15 2:50 μl) from Immunotools. The isotype controls PE-labeled IgG_{2A} (clone 54447), FITC-labeled IgG_{2B} (clone 141945), APC-labeled IgG_{2A} (54447) and Alexa Fluor®488-labeled IgG, all from R&D Systems and APC-labeled IgG2b from Immunotools, were used as negative controls to define

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