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Biodegradable chitosan particles induce chemokine release and negligible arginase-1 activity compared to IL-4 in murine bone marrow-derived macrophages

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ABSTRACT

Alternatively activated macrophages have been implicated in the therapeutic activity of biodegradable chitosan on wound healing, however, the mechanisms of phenotypic differentiation are still unclear. *In vitro*, macrophages stimulated with high doses of chitosan (⩾500 μg/mL) were reported to produce low-level markers associated with alternative activation (arginase-1) as well as classical activation (nitric oxide), and to undergo apoptosis. In this study, we tested the hypothesis that 40 kDa biodegradable chitosan (5–500 μg/mL) is sufficient to polarize mouse bone marrow-derived macrophages (BMDM) *in vitro* to an alternatively activated phenotype. Control cultures were stimulated with IL-4 (alternative activation), IFN-γ/LPS (classical activation), 1 μm diameter latex beads (phagocytosis), or left untreated. After 48 h of *in vitro* exposure, BMDM phagocytosed fluorescent chitosan particles or latex beads, and remained viable and metabolically active, although some cells detached with increasing chitosan and latex bead dosage. Arginase-1 was over 100-fold more strongly induced by IL-4 than by chitosan, which induced only sporadic and weak arginase-1 activity over untreated BMDM, and no nitric oxide. IFN-γ/LPS stimulated nitric oxide production and arginase-1 activity and high concentrations of inflammatory cytokines (IL-6, IL-1β, TNF-α, MIP-1α/MIP-1β), while latex beads stimulated nitric oxide and not arginase-1 activity. Chitosan or latex bead exposure, but not IL-4, tended to promote the release of several chemokines (MIP-1α/β, GM-CSF, RANTES, IL-1β), while all treatments promoted MCP-1 release. These data show that chitosan phagocytosis is not sufficient to polarize BMDM to the alternative or the classical pathway, suggesting that biodegradable chitosan elicits alternatively activated macrophages *in vivo* through indirect mechanisms.

1. Introduction

Macrophages play an important role in wound repair through the local release of chemotactic, inflammatory, and angiogenic cytokines [1]. Three macrophage populations have been previously identified based on the stimuli they received *in vitro*. Macrophages primed with interferon-γ (IFN-γ) and stimulated with lipopolysaccharide (LPS) are described as classically activated macrophages (M1 MΦ); type II-activated or regulatory MΦ can be generated by immune complexes, prostaglandins, glucocorticoids, and IL-10; and alternatively activated macrophages (AA MΦ) or wound-repair MΦ are obtained by IL-4 or IL-13 stimulation [2,3]. One of the distinguishing characteristics between murine M1 and AA MΦ is their metabolic pathway for L-arginine. While M1 MΦ promote inflammation through nitric oxide (NO) production by nitric oxide synthases, AA MΦ promote wound healing through the arginase-1-mediated production of polyamines and proline [2,4].

Chitosan is a linear biocompatible polysaccharide used in tissue engineering that is known to attract MΦ *in vitro* and *in vivo* [5,6]. Chitosan is mainly composed of glucosamine with variable levels of *N*-acetyl-D-glucosamine (GlcNAc); when GlcNAc content approaches 20% (~80% degree of deacetylation, DDA), the polymer is biodegradable. In a rabbit marrow stimulation model of cartilage repair, chitosan hybrid blood clot implants promoted transient accumulation of arginase-1+ MΦ, followed by angiogenesis and improved subchondral bone and cartilage repair [7–9]. These data suggest that AA MΦ may serve a therapeutic and essential role in chitosan-stimulated wound repair, but the mechanisms of alternative activation are incompletely understood. Porporatto et al. [10]

Abbreviations: BMDM, bone marrow-derived macrophages; MΦ, macrophage; M1 MΦ, classically activated macrophages; AA MΦ, alternatively activated macrophages; IFN-γ, interferon-γ; LPS, lipopolysaccharide; IL-4, interleukin-4; NO, nitric oxide; RITC, rhodamine B isothiocyanate; CM, conditioned media.

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showed that low molecular weight chitosan (~80% DDA, 50 kDa, 0.5–1 mg/mL) stimulated a 2–3-fold increase in arginase-1 activity as well as NO activity, in cultured rat peritoneal resident MΦ. However, in parallel cultures, high molecular weight 80% DDA chitosan stimulated NO activity and not arginase-1 [10]. Chitin, the parent molecule of chitosan (100% GlcNA) elicited arginase-1+ MΦ to mouse lung [11], but did not stimulate appreciable arginase-1 gene expression in cultured bone marrow-derived macrophages (BMDM) [12]. Phagocytosis could play a role in MΦ polarization since both chitosan particles and latex beads stimulated TGF-β1 and PDGF release from *in vitro* cultured MΦ [13]. At *in vitro* concentrations $\geqslant$0.5 mg/mL however, chitosan has cytotoxic effects on monolayer cells [14], and induces apoptosis of purified adherent macrophages [15]. Altogether, these data suggest that biodegradable chitosan particles could directly polarize MΦ to an alternatively activated phenotype if applied to cells at cytocompatible concentrations (i.e., $\leqslant$500 μg/mL).

To-date, most of the studies analyzing the effect of chitosan on MΦ activation have used peritoneal or blood-derived MΦ [5,10,13,15]. In the context of bone marrow-derived cartilage repair, MΦ derived from bone marrow could be considered closer to the cell types involved in osteochondral repair. Here, we tested the hypothesis that low molecular weight biodegradable chitosan particles directly induce alternative activation of murine BMDM, using arginase-1 as a marker of AA MΦ. IL-4 served as a positive control [2], IFN-γ/LPS as a control for M1 MΦ activation [2], and latex beads served as a control for phagocytosis.

2. Materials and methods

2.1. Materials

Chitosans having 81.9% and 80.0% degree of deacetylation (DDA), Mn 33.0 and 37.9 kDa, and polydispersity 2.4 and 2.6 were obtained by nitrous acid depolymerization of an ultrapure chitosan (Mn ~150 kDa, 80% DDA,<500 EU/g, BioSyntech Inc. Laval, QC, Canada). Rhodamine B isothiocyanate–chitosan (RITC–chitosan), 81.3% DDA, Mn 48.5 kDa, polydispersity 5.4, with 0.5% mol/mol RITC/chitosan was prepared as previously described [16]. Fluorescent latex beads mean diameter 1 μm (carboxylate-modified polystyrene yellow-green), lipopolysaccharide (LPS), Dulbecco's Phosphate Buffered Saline (D-PBS) calcium-magnesium free, ethylenediaminetetraacetic acid (EDTA) tetrasodium salt, bovine serum albumin, Triton X-100, aprotinin, urea, Tris–HCl, $MnCl_2$, L-arginine, H_3PO_4, and alpha-isonitrosopropiophenone were purchased from Sigma–Aldrich (Oakville, ON, Canada). Recombinant IL-4 and IFN-γ were from R&D Systems (Cedarlane, Hornby, ON, Canada). Pepstatin A and antipain were purchased from Calbiochem-EMD (Merck KGaA, Darmstadt, Germany).

2.2. Preparation of bone marrow derived macrophages

All animal experimental protocols were approved by an institutional animal care committee. Murine bone marrow-derived macrophages (BMDM) were prepared as described previously [17–19]. Briefly, two 8-week-old male BALB/c mice per cell isolation were euthanized by CO_2, and bone marrow cells were immediately flushed from the aseptically dissected tibias and femurs using cold sterile RPMI. Pooled cells were allowed to adhere to plastic at 37 °C for 2 h under standard incubation conditions, and non-adherent cells were recovered and seeded in 24-well plates at 5×10^5 cells/well. Cells were grown for 7 days in RPMI-1640 (Gibco, Invitrogen, Burlington, ON, Canada) with 10% (v/v) heat-inactivated fetal bovine serum (HiFBS, Atlanta Biologicals, Atlanta, GA, USA), 100 U/mL penicillin, and 100 μg/mL streptomycin (Sigma–Aldrich), supplemented with 15% (v/v) L929-cell conditioned medium (L929-CM), at 37 °C and 5% CO_2, with media changes every 3 days. L929-CM was derived from 5- to 6-day supernatants of confluent L929 cells grown in 10% HiFBS-αMEM (cleared of cell debris by centrifugation, filtered with a 0.2 μm filter and stored frozen at −20 °C) as a source of macrophage colony-stimulating factor (M-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF) [20].

2.3. Treatment of BMDM

After 7 days of differentiation, the media was refreshed, and BMDM were either left untreated or treated for 48 h with filter-sterile chitosan (5, 50, 100, and 500 μg/mL), latex beads (50 and 500 μg/mL), mouse recombinant IL-4 (0.5 ng/mL), or with 18 h of previous priming with mouse recombinant IFN-γ (12 ng/mL) and post-treatment for 48 h with LPS from *Salmonella* (10 ng/mL). Following treatment, cells were detached with 2 mM EDTA (4 °C, 15 min), counted, and 10^5 cells were lysed (50 μL of 0.1% v/v Triton X-100 in the presence of protease inhibitors) for arginase-1 assays; while conditioned media were collected cleared of debris by centrifugation (1300 rpm, 10 min, 4 °C) and frozen in separate aliquots at −80 °C for the different analyzes. Thawed conditioned media samples were further centrifuged at 13,200 rpm for 10 min at 4 °C prior to analysis of cytokine and chemokine release.

2.4. Confocal microscopy

BMDM differentiated after 7 days on plastic round sterile coverslips (Sarstedt, Cedarlane, Hornby, ON, Canada) were left untreated or were treated with latex beads (50 μg/mL) or RITC–chitosan particles (50 and 500 μg/mL) for 48 h. Cells were then incubated for 5 min at 37 °C in serum-free RPMI containing 2.5 μg/mL Cell Mask™ deep red plasma membrane stain (Invitrogen, Burlington, ON, Canada), or for 30 min at 37 °C in serum free RPMI containing 0.4 μM calcein AM (viable green cytosol; Molecular Probes, Invitrogen). The labeling media were replaced by RPMI, and live confocal imaging was performed with a LSM 510 META Axioplan 2 confocal scanning microscope equipped with an apochromat 63×/0.9 NA water immersion objective (Carl Zeiss, Germany). Images were captured from representative areas of each sample after scanning through the z-axis.

2.5. Cell metabolic activity: lactate/glucose quantification and alamar blue assay for redox activity

After 48 h of BMDM treatment, D-glucose consumption and L-lactate production in the BMDM-conditioned media were measured by a dual-channel immobilized oxidase enzyme biochemistry analyzer (2700 SELECT, YSI Inc. Life Sciences, Yellow Springs, OH, USA), using a lactate calibration buffer provided by the manufacturer.

Alamar blue (Invitrogen) was added to each well and incubated for 4 h at 37 °C. Eighty microliter of media containing reduced alamar blue dye (due to metabolic activity) was read at excitation 560 nm, emission 590 nm and cut off 570 nm as described [21]. The relative oxidation–reduction (redox) activity (%) compared to untreated BMDM was calculated by the following equation $[\text{Fluorescence}]_{\text{sample}} / [\text{Fluorescence}]_{\text{control}} \times 100$.

2.6. Arginase-1 assay

Macrophage arginase activity was quantified in cell lysates by urea determination with α-isonitrosopropiophenone, based on a modification of Schimke's method [22]. Briefly, after enzyme activation, L-arginine was hydrolyzed for 2 h, and the resultant urea in