



# Ca<sup>2+</sup> released from calcium alginate gels can promote inflammatory responses *in vitro* and *in vivo*



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## ABSTRACT

In general, alginate hydrogels are considered to be biologically inert and are commonly used for biomedical purposes that require minimum inflammation. However, Ca<sup>2+</sup>, which is commonly used to crosslink alginate, is a critical second messenger in immune cell signaling, and little has been done to understand its effect on immune cell fate when delivered as a component of alginate gels. We found that dendritic cells (DCs) encapsulated in Ca<sup>2+</sup>-crosslinked alginate (calcium alginate) secreted at least fivefold more of the inflammatory cytokine IL-1 $\beta$  when compared to DCs encapsulated in agarose and collagen gels, as well as DCs plated on tissue-culture polystyrene (TCPS). Plating cells on TCPS with the alginate polymer could not reproduce these results, whereas culturing DCs on TCPS with increasing concentrations of Ca<sup>2+</sup> increased IL-1 $\beta$ , MHC class II and CD86 expression in a dose-dependent manner. In agreement with these findings, calcium alginate gels induced greater maturation of encapsulated DCs compared to barium alginate gels. When injected subcutaneously in mice, calcium alginate gels significantly upregulated IL-1 $\beta$  secretion from surrounding tissue relative to barium alginate gels, and similarly, the inflammatory effects of LPS were enhanced when it was delivered from calcium alginate gels rather than barium alginate gels. These results confirm that the Ca<sup>2+</sup> used to crosslink alginate gels can be immunostimulatory and suggest that it is important to take into account Ca<sup>2+</sup>'s bioactive effects on all exposed cells (both immune and non-immune) when using calcium alginate gels for biomedical purposes. This work may strongly impact the way people use alginate gels in the future as well as provide insights into past work utilizing alginate gels.

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## 1. Introduction

It is widely appreciated that Ca<sup>2+</sup> is one of the most common second messengers in cell signaling, with important roles in transcription, apoptosis, cell adherence, activation, exocytosis, metabolism and proliferation [1–3]. White blood cells are examples of cells that are highly dependent on Ca<sup>2+</sup> signaling for their function. For instance, dendritic cells (DCs) require Ca<sup>2+</sup> signaling for cytokine secretion, maturation marker expression and phagocytosis [4,5], mast cells and neutrophils require calcium for degranulation and T cells require Ca<sup>2+</sup> signaling for the production of IL-2 and IL-4 [6–8]. Calcium's importance in the proper functioning of the immune system can be underscored by the fact that a single missense mutation in the gene encoding the Ca<sup>2+</sup> release-activated Ca<sup>2+</sup> channel, an important Ca<sup>2+</sup> channel in the plasma membrane expressed by a number of immune cells, knocks out its function,

causing severe combined immunodeficiency in humans [6]. Because of Ca<sup>2+</sup>'s importance in immune cell function, it has been proposed that Ca<sup>2+</sup> channels and Ca<sup>2+</sup> signaling pathways are promising therapeutic targets to control immune cell behavior [4–6,8,9].

Alginate, also known as alginic acid, is an anionic, linear and unbranched polysaccharide isolated from algae or bacterial biofilms. Alginate is composed of (1, 4)-linked,  $\beta$ -D-mannuronate (M) and  $\alpha$ -L-guluronate (G) sugar monomers that are arranged in M blocks (MMMMMM), G blocks (GGGGGG) or alternating M and G residues (MGMGMG), with the exact M and G composition being dependent on the algae or bacteria source. Alginate polymers have a high affinity for divalent cations (in the order  $Mg^{2+} \ll Ca^{2+} < Sr^{2+} < Ba^{2+}$ ) and can form a crosslinked network when these divalent cations associate with the G blocks in a proposed “egg-box” model to form crosslinks between the polymer chains [10,11]. Thus, alginate polymers rich in G blocks are able to create more ionic crosslinks and stiffer gels [10,12]. Ca<sup>2+</sup>-crosslinked alginate gels (calcium alginate) encapsulating growth factors, cells and/or cytokines have been used *in vivo* for a wide variety of applications such as type I diabetes treatment [13] and bone regeneration [14]. Interestingly,

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in a study where  $\text{Ca}^{2+}$ -crosslinked alginate gels were used to deliver pro-angiogenic factors to enhance blood vessel formation [15], and in another study where they were used to deliver activated dendritic cells peritumorally to reduce tumor growth [16], alginate gels alone appeared to have a slight therapeutic effect, but none of these studies specifically examined the potential contribution of  $\text{Ca}^{2+}$  to the final outcome.

Contrary to the lack of studies examining the effects of calcium crosslinker, the inflammatory properties of alginate polysaccharides have been widely studied and disputed. For example, dissolved alginate polysaccharides ( $100\text{--}1000\ \mu\text{g ml}^{-1}$ ) have been shown to activate monocytes and macrophages, depending on the molecular weight and the M and G ratio of the polymer [17–19]. However, other studies have shown that alginate polysaccharides can actually suppress inflammatory disease [20] or have demonstrated no effect at all [21].

Based on the importance of  $\text{Ca}^{2+}$  signaling in white blood cell activation, we hypothesized that the  $\text{Ca}^{2+}$  released from calcium alginate gels could promote inflammatory responses *in vitro* and *in vivo*. For *in vitro* studies, DCs were tested as a model leukocyte given their importance in dictating immune responses. To evaluate the immunostimulatory effects of alginate gels, calcium alginate was tested against three commonly used biomaterials (agarose, collagen and tissue culture polystyrene (TCPS)) for its ability to induce DC maturation and/or affect LPS-induced activation *in vitro*. The impact of both the alginate polymer itself and the  $\text{Ca}^{2+}$  used to crosslink the alginate gels was assessed. The cytokines IL-1 $\beta$ , IL-4, IL-6, IL-10, IL-12p70, IFN- $\gamma$  and TNF- $\alpha$  and the activation markers CD86 and MHC class II were analyzed to gauge DC maturation. For *in vivo* studies,  $\text{Ca}^{2+}$ - or  $\text{Ba}^{2+}$ -crosslinked alginate gels, with or without LPS, were injected subcutaneously into C57BL/6J mice to determine their ability to induce local inflammatory cytokine secretion from surrounding tissue.

## 2. Materials and methods

### 2.1. Cell culture

Dendritic cells were generated from bone marrow isolated from 4–16 week old C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME) as described by Lutz et al. [22].

### 2.2. Endotoxin testing

All polymer solutions, calcium crosslinkers and other ion-supplemented solutions used in this study were tested using the Limulus Amebocyte Lysate (LAL) Assay (Lonza, Walkersville, MD) according to the manufacturer's instructions.

### 2.3. Comparing DC activation across TCPS, collagen, agarose and $\text{CaSO}_4$ -crosslinked alginate gels

For TCPS conditions, DCs were plated at a concentration of 250,000 cells/100  $\mu\text{l}$  phosphate buffered saline (PBS)/well of a 48-well plate and incubated at 37 °C for 30 min. For collagen gel fabrication, rat tail collagen, type I (BD Biosciences, Franklin Lakes, NJ) was certified to be negative for bacteria, fungi and mycoplasma and used without further purification. DCs were harvested, washed once in PBS and resuspended at a concentration of  $10 \times 10^6$  cells  $\text{ml}^{-1}$  in a 3 mg  $\text{ml}^{-1}$  ice cold collagen solution prepared aseptically according to the manufacturer's instructions. 100  $\mu\text{l}$  ( $10^6$  cells) were pipetted into the wells of a 48-well plate and allowed to cure at 37 °C for 30 min. For agarose gel fabrication, a 1.2% Sea-Plaque<sup>®</sup> agarose solution (Lonza, Allendale, NJ) in PBS was sterilized by autoclaving. For cell encapsulation, the solution was

microwaved until fully dissolved. After cooling to 40 °C in a water bath, DCs were resuspended in agarose at a concentration of  $10 \times 10^6$  cells  $\text{ml}^{-1}$ , and 100  $\mu\text{l}$  ( $10^6$  cells) were immediately pipetted into wells of a 48-well plate. The agarose quickly cured at room temperature and was placed at 37 °C for 30 min. For alginate gel fabrication, PRONOVA<sup>™</sup> Ultrapure medium viscosity alginate rich in  $\alpha$ -L-guluronate residues (MVG) (FMC BioPolymer, Sandvika, Norway) was certified to be free of yeast, mold and bacteria and have an endotoxin content  $\leq 100\ \text{EU g}^{-1}$ . MVG alginate was further sterilized by dissolving it in deionized water and filtering it through a 0.22  $\mu\text{m}$  pore diameter membrane (Millipore, Billerica, MA). The sterile alginate solution was frozen, lyophilized and reconstituted aseptically in PBS to make a 2% solution. A  $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$  slurry (183 mM) in deionized water was sterilized by autoclaving. DCs in PBS were mixed with the 2% alginate solution using two 1 ml syringes connected with a nylon female luer thread style coupler (Value Plastics, Fort Collins, CO) for a final concentration of  $13 \times 10^6$  cells  $\text{ml}^{-1}$ . 78  $\mu\text{l}$  ( $10^6$  cells) of this suspension was added to wells of a 48-well plate using an 18 gauge needle, and 22  $\mu\text{l}$  of thoroughly mixed  $\text{CaSO}_4$  slurry was quickly pipetted and stirred into the alginate in each well. The alginate was allowed to cure at 37 °C for 30 min. Final alginate gels contained 1.2% alginate, 40 mM  $\text{CaSO}_4$  and  $10^6$  cells in a 100  $\mu\text{l}$  gel volume.

After DC plating and encapsulation in hydrogels, 350  $\mu\text{l}$  of R10 medium was added to each well and allowed to equilibrate at 37 °C and 5%  $\text{CO}_2$  for 1 h before activation. For activation, 50  $\mu\text{l}$  of 1000 ng  $\text{ml}^{-1}$  LPS (*E. coli* 0111:B4; Sigma–Aldrich) in R10 was added to each well so that the final concentration was 100 ng  $\text{ml}^{-1}$ ; for LPS-free wells, 50  $\mu\text{l}$  of R10 only was added. After 20–24 h of activation on an orbital shaker, supernatant was collected and frozen at  $-20\ ^\circ\text{C}$  for cytokine analysis.

### 2.4. Soluble polymer studies with collagen, agarose and alginate

Sterile 1 mg  $\text{ml}^{-1}$  solutions of dissolved collagen, agarose and alginate were made in PBS. In addition to the polymers used for gel fabrication, additional alginate polymers tested included pure G and M blocks (provided by Dr. Kamal Bouhadir at the American University of Beirut, Lebanon) and PRONOVA<sup>™</sup> Ultrapure medium viscosity (MV), low viscosity (LV) and very low viscosity (VLV) alginates rich in either G or M residues (FMC BioPolymer). Each polymer solution was then diluted 1:10 in R10 medium for a final concentration of 100  $\mu\text{g ml}^{-1}$  (referred to as polymer R10). PBS was used for the control. To prevent any potential differences in protein adsorption from affecting cell attachment across the different conditions, 200  $\mu\text{l}$  of R10 medium was added to wells of a 96-well plate and incubated overnight to coat the wells with serum proteins prior to cell plating. DCs were harvested, washed in PBS, resuspended in the 100  $\mu\text{g ml}^{-1}$  polymer solutions above and plated at a density of 100,000 cells/180  $\mu\text{l}$ /serum-coated well. After 1 h of incubation at 37 °C and 5%  $\text{CO}_2$ , cells were stimulated with 20  $\mu\text{l}$  of 1000 ng  $\text{ml}^{-1}$  LPS in basal R10 or polymer R10 to give a final concentration of 100 ng  $\text{ml}^{-1}$ ; for LPS-free wells, 20  $\mu\text{l}$  of basal R10 or polymer R10 only was added. After 20–24 h, supernatant was collected and frozen at  $-20\ ^\circ\text{C}$  for cytokine analysis.

### 2.5. Soluble $\text{Ca}^{2+}$ dose studies

Sterile, high  $\text{Ca}^{2+}$ -containing medium was made by adding  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  to deionized water, filtering it through a 0.22  $\mu\text{m}$  membrane and diluting it 1:100 in R10 so that the final  $\text{Ca}^{2+}$  concentration ( $[\text{Ca}^{2+}]$ ) equaled 3, 6 or 12 mM (basal R10 = 0.42 mM). Sterile deionized water was used for the control. To confirm that results were not due to a  $\text{Ca}^{2+}$  artifact,  $\text{Mg}^{2+}$ ,  $\text{K}^+$ ,  $\text{Na}^+$  and A23187 (Sigma–Aldrich), a  $\text{Ca}^{2+}$  ionophore that increases intracellular  $\text{Ca}^{2+}$  concentration ( $[\text{Ca}^{2+}]_i$ ), were also screened for their ability

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