Published in final edited form as:
Acta Biomater. 2013 December ; 9(12): . doi:10.1016/j.actbio.2013.08.002.

Ca^{2+} 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^{2+}, 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^{2+}-crosslinked alginate (calcium alginate) secreted at least fivefold more of the inflammatory cytokine IL-1β 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^{2+} increased IL-1β, 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-1β 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^{2+} used to crosslink alginate gels can be immunostimulatory and suggest that it is important to take into account Ca^{2+}'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.

Keywords

Alginate; Ca^{2+} signaling; Dendritic cell; Tissue engineering; IL-1β

1. Introduction

It is widely appreciated that Ca^{2+} 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^{2+} signaling for their function. For instance, dendritic cells (DCs) require Ca^{2+} signaling for cytokine secretion, maturation marker expression and phagocytosis [4,5], mast cells and neutrophils require calcium for degranulation and T cells require Ca^{2+} 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

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missense mutation in the gene encoding the Ca^{2+} release-activated Ca^{2+} channel, an important Ca^{2+} 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^{2+}'s importance in immune cell function, it has been proposed that Ca^{2+} channels and Ca^{2+} 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, β-D-mannuronate (M) and α-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^{2+}-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, in a study where 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 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 (100–1000 $\mu g\ ml^{-1}$) 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 Ca^{2+} signaling in white blood cell activation, we hypothesized that the 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 Ca^{2+} used to crosslink the alginate gels was assessed. The cytokines IL-1β, IL-4, IL-6, IL-10, IL-12p70, IFN-γ and TNF-α and the activation markers CD86 and MHC class II were analyzed to gauge DC maturation. For *in vivo* studies, Ca^{2+}- or Ba^{2+}-crosslinked alginate gels, with or without LPS, were injected subcutaneously into C576BL/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 $CaSO_4$-crosslinked alginate gels

For TCPS conditions, DCs were plated at a concentration of 250,000 cells/100 μl 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 10×10^6 -cells ml^{-1} in a 3 mg ml^{-1} ice cold collagen solution prepared aseptically according to the manufacturer's instructions. 100 μl (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® 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 10×10^6 cells ml^{-1}, and 100 μl (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™ Ultrapure medium viscosity alginate rich in α-L-guluronate residues (MVG) (FMC BioPolymer, Sandvika, Norway) was certified to be free of yeast, mold and bacteria and have an endotoxin content ≤100 EU g^{-1}. MVG alginate was further sterilized by dissolving it in deionized water and filtering it through a 0.22 μm pore diameter membrane (Millipore, Billerica, MA). The sterile alginate solution was frozen, lyophilized and reconstituted aseptically in PBS to make a 2% solution. A $CaSO_4 \cdot 2H_2O$ 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 13×10^6 cells ml^{-1}. 78 μl (10^6cells) of this suspension was added to wells of a 48-well plate using an 18 gauge needle, and 22 μl of thoroughly mixed $CaSO_4$ 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 $CaSO_4$ and 10^6 cells in a 100 μl gel volume.

After DC plating and encapsulation in hydrogels, 350 μl of R10 medium was added to each well and allowed to equilibrate at 37°C and 5% CO_2 for 1 h before activation. For activation, 50 μl of 1000 ng ml^{-1} LPS (*E. coli* 0111:B4; Sigma–Aldrich) in R10 was added to each well so that the final concentration was 100 ng ml^{-1}; for LPS-free wells, 50 μl of R10 only was added. After 20–24 h of activation on an orbital shaker, supernatant was collected and frozen at −20 °C for cytokine analysis.

2.4. Soluble polymer studies with collagen, agarose and alginate

Sterile 1 mg 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™ 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 μg 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 μl 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 μg ml^{-1} polymer solutions above and plated at a density of 100,000 cells/180 μl/serum-coated well. After 1 h of incubation at 37 °C and 5% CO_2, cells were stimulated with 20 μl of 1000 ng ml^{-1} LPS in basal R10 or polymer R10 to give a final concentration of 100 ng ml^{-1}; for LPS-free wells, 20 μl of basal R10 or polymer R10 only was added. After 20–24 h, supernatant was collected and frozen at −20 °C for cytokine analysis.

2.5. Soluble Ca^{2+} dose studies

Sterile, high Ca^{2+}-containing medium was made by adding $CaCl_2 \cdot 2H_2O$ to deionized water, filtering it through a 0.22 μM membrane and diluting it 1:100 in R10 so that the final Ca^{2+} concentration ($[Ca^{2+}]$) 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 Ca^{2+} artifact, Mg^{2+}, K^+, Na^+ and A23187 (Sigma–Aldrich), a Ca^{2+} ionophore that increases intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), were also screened for their ability to alter cytokine expression. Medium was supplemented with 400 ng ml^{-1} A23187 or each ion so that its final concentration ($[Ca^{2+}]$ = 3 mM; $[Mg^{2+}]$ = 1 mM; $[K^+]$ = 5.6 mM, $[Na^+]$ = 162 mM) was 10% higher than the physiological upper limit. DCs were harvested, washed once in PBS and resuspended in basal medium or R10 containing high Ca^{2+}, Mg^{2+}, K^+, Na^{2+} or A23187 (referred to as high ion R10). For cytokine analysis, 100,000 cells/180 μl of basal or high ion R10 were plated per well of a 96-well plate and allowed to equilibrate at 37 °C and 5% CO_2. After 1 h, 20 μl of 1000 ng ml^{-1} LPS in basal or high ion R10 was added to each well for a final concentration of 100 ng ml^{-1}; for LPS-free wells, 20 μl of basal or high ion R10 only was added. After 20–24 h of activation supernatantwas collected and frozen at −20 °C. To measure surface marker expression, 10^6 cells/1.8 ml basal or high calcium R10 were plated per well of a 6-well plate and stimulated with 200 μl of activation or LPS-free medium as above. Cytokines and cell-surface markers were detected and analyzed as described below.

2.6. Comparing the effect of $BaCl_2$-crosslinked, $CaCl_2$-crosslinked and $CaSO_4$-crosslinked alginate gels in vitro

To compare DCs encapsulated within barium and calcium alginate gels, DCs were harvested, washed once in PBS and resuspended in R10 at a concentration of 125×10^6 cells ml^{-1}. 800 μl of a sterile 2.5% Ultrapure MVG alginate solution in PBS was mixed with 200 μl of cell suspension using two 5 ml syringes connected with a coupler to give a final concentration of 25×10^6 cells ml^{-1} of 2% alginate. A sterile beaker containing a sterile 10 mM $BaCl_2$ or 100 mM $CaCl_2$ solution with 0.1 M HEPES in ddH_2O (pH 7.4) was stirred using a stir plate while the alginate was ejected into the bath using a 30 gauge needle to create uniform alginate beads ~2 mm in diameter (~4 μl volume) with ~100,000 cells per bead. The beads were allowed to stir for 10 min for complete gelation, poured into a 0.22 μM filter and washed twice with 50 ml of PBS. Ten beads (~10^6 cells) were placed in each well of a 48-well plate containing 500 μl medium and were allowed to equilibrate at 37 °C and 5% CO_2. After 1 h, 60 μl of 1000 ng ml^{-1} LPS in R10 was added so that the final concentration in each well was 100 ng ml^{-1}; for LPS-free wells, 60 μl of R10 only was added. Beads were incubated on an orbital shaker for 20–24 h, after which supernatant was collected and frozen at −20 °C. For cytokine and cell-surface marker analysis, see below.

To compare DCs encapsulated in beads crosslinked with 10 or 100 mM $CaCl_2$, DCs were harvested, washed once in PBS and resuspended in PBS at a concentration of 100×10^6 cells ml^{-1}. 750 μl of a sterile 2% Ultrapure MVG alginate solution in R10 was mixed with 250 μl of cell suspension using two 5 ml syringes connected with a coupler to give a final concentration of 25×10^6 cells ml^{-1} of 1.5% alginate. A sterile beaker containing a sterile

10 or 100 mM $CaCl_2$ solution with 0.1 M HEPES in ddH_2O (pH 7.4) was stirred using a stir plate while the alginate was ejected into the $CaCl_2$ bath using a 30 gauge needle to create uniform alginate beads ~2 mm in diameter (~4 μl volume) with ~100,000 cells per bead. The beads were washed and cultured as above.

To compare DCs cultured in 10 or 40 mM $CaSO_4$-crosslinked alginate discs, DCs were harvested, washed once in PBS and resuspended in R10 at a concentration of 128×10^6 cells ml^{-1}. 1.2 ml of a sterile 2% Ultrapure MVG alginate solution in R10 was mixed with 300 μl of cell suspension using two 3 ml syringes connected with a coupler. The syringe containing the alginate–cell suspension was then connected to a third syringe containing 420 μl of 183 or 46 mM $CaSO_4$ crosslinker. The two syringes were pumped back and forth quickly 8–10 times to homogeneously mix the alginate with the crosslinker, and the mixture was cast between two Teflon-coated aluminum plates (McMaster-Carr, Elmhurst, IL) with 1 mm spacers. The alginate was allowed to cure for 10–20 min, after which 8 mm diameter discs were punched using a disposable biopsy punch (Premier, Plymouth Meeting, PA). Final discs were 8 × 1 mm (~50 μl) and contained 1.25% alginate, 10^6 cells and either 10 or 40 mM $CaSO_4$. Discs were placed in 490 μl R10 in 48-well plates and allowed to equilibrate at 37 °C and 5% CO_2. After 1 h, 60 μl of 1000 ng ml^{-1} LPS in R10 was added so that the final concentration in each well was 100 ng ml^{-1}; for LPS-free wells, 60 μl of R10 only was added. Discs were incubated on an orbital shaker for 20–24 h after which supernatant was collected and frozen at −20 °C.

To compare DCs cultured externally from gels, 10^6 DCs in 2 ml of medium were plated in 6-well plates. After 1 h of culture, 200 μl of Ba^{2+}- or Ca^{2+}-crosslinked alginate gels were added to wells. Gels were fabricated by mixing a 2.5% MVG alginate solution in R10 with 20 mM $BaCl_2$ or 244 mM $CaSO_4$ crosslinker solution in water in a 4:1 ratio using two syringes connected by a coupler. 200 μl was ejected through an 18 gauge needle onto a sterile plate and allowed to cure for 10 min. Final gels, containing 2% alginate and 4 mM barium or 48.8 mM calcium, were then added to wells using a sterile spatula. After 20–24 h of stagnant culture, supernatants were collected for IL-1β analysis. For surface marker analysis, the above procedure was repeated, except that gels were cast between two Teflon-coated aluminum plates and 8 mm discs were punched using a biopsy punch. Four gels were added per well and DCs were cultured on an orbital shaker. After 20–24 h, cells were scraped from wells for surface marker staining as described below.

2.7. In vivo studies

Female C57BL/6 J mice (Jackson Laboratory, Bar Harbor, ME), aged 4–16 weeks, were anesthetized with isoflurane, and their backs were shaved and wiped with ethanol. A 2.5% MVG alginate solution in PBS was mixed with a sterile 20 mM $BaCl_2$ or 244 mM $CaSO_4$ solution in water using two 1 ml syringes connected by a coupler. The volumes were mixed in a 4:1 ratio so that the final gel contained 2% alginate and 4 mM $BaCl_2$ or 48.8 mM $CaSO_4$. For alginate gels delivering LPS, MVG alginate was dissolved with PBS containing LPS so that final crosslinked gels contained 1 μg of LPS/50 μl gel. All gels were prepared aseptically. After mixing the dissolved alginate with the crosslinker, 50 μl of gel was injected subcutaneously with a 23 gauge needle in the center of the back. Mice were allowed to recover and consume food and water *ad libitum*. At the timepoints specified, mice were sacrificed and gels were removed. Samples were frozen at −20 °C until analysis. These studies were performed in compliance with the NIH Guide for Care and Use of Laboratory Animals and the Guidelines for the Use of Vertebrate Animals in Research and Teaching of the Faculty of Arts and Sciences of Harvard University.

2.8. Cell-surface marker analysis

For studies on TCPS, 1 ml of 50 mM EDTA in PBS (pH 7.4) was added to each well, and plates were placed in the incubator for 15 min to aid in cell detachment. Cells were then scraped from wells and washed with stain buffer (BD Pharmingen, Franklin Lakes, NJ), which contained standard PBS and 0.2% BSA. To retrieve DCs from the gels, 500 μl of 50 mM EDTA in PBS (pH 7.4) was added to each well. Gels were allowed to dissolve at 37 °C and cells were collected and washed in stain buffer. Collected cells were stained with APC-conjugated anti-mouse CD11c, FITC-conjugated anti-mouse MHC class II and PE-conjugated anti-mouse CD86 (eBioscience, San Diego, CA). Cell-surface antigen staining was analyzed using an LSR II or LSR Fortessa™ flow cytometer (BD Biosciences). Cell viability was determined using forward-(FSC) and side-scatter (SSC), and only viable cells were gated for surface marker analysis. A Live/Dead® Violet Fixable Dead Cell Stain Kit (Life Technologies) was used according to the manufacturer's instructions to confirm cell viability.

2.9. Cytokine analysis

Cell culture supernatants were analyzed for mouse IL-1β, IL-4, IL-6, IL-10, IL-12p70, IFN-γ and TNF-α using the Bio-Plex Pro™ Magnetic Cytokine Assay System (Bio-Rad, Hercules, CA) or mouse IL-1β only using an Quantikine® Colorimetric Sandwich ELISA kit (R&D Systems, Minneapolis, MN). For *in vivo* assays, gels were digested with 100 μl of 10 unit ml^{-1} alginate lyase (Sigma) in a 37°C dry bath with occasional vortexing until fully dissolved. Digested alginate was analyzed with the Bio-Plex Pro™ Mouse Cytokine 23-plex Assay System and with an IL-1β Quantikine® Colorimetric Sandwich ELISA kit. An IL-1β control was resuspended in deionized H_2O or deionized H_2O supplemented with 12 mM Ca^{2+} and assayed via ELISA to confirm that high levels of Ca^{2+} in the supernatants were not affecting antibody detection of IL-1β.

2.10. Ca^{2+} release assay

For *in vitro* studies, alginate beads and discs were fabricated and incubated in R10 as above but without cells. Medium was collected at 1, 4 and 10 h, frozen at −20 °C. For *in vivo* studies, gels were harvested at the timepoints indicated, digested with alginate lyase and diluted 1:4 in PBS. All samples were assayed using the QuantiChrom™ Calcium Assay Kit (BioAssay Systems, Hayward, CA) according to the manufacturer's instructions. Absorbance was read with a Synergy™ HT microplate reader (Bio-Tek, Winooski, VT).

2.11. Intracellular Ca^{2+} assay

To measure $[Ca^{2+}]_i$, cells were labeled with the fluorescent intracellular Ca^{2+} probe Fluo-4 AM (Life Technologies). DCs were harvested, washed in PBS and resuspended at a density of 10^6 cells ml^{-1} in 5 μM Fluo-4 AM in PBS for 30 min at room temperature. Labeled cells were then washed in PBS and allowed to sit for another 30 min to allow for complete de-esterification of the probe before plating. Immediately after plating, cells were imaged with an EVOS® fl microscope (AMG, Bothell, WA) using the FITC channel. For kinetic studies, DCs were plated on TCPS in increasing $[Ca^{2+}]$ as above and fluorescence was measured using a Synergy™ HT microplate reader (Ex. 488 nm, Em. 516) at multiple timepoints after plating. For all Fluo-4 studies, the anti-FITC antibody A889 (Life Technologies) was diluted in the medium 1:200 to quench background fluorescence.

2.12. Differential interference contrast (DIC) microscopy and photos of whole alginate gels

DIC images of cells were taken using an Olympus IX81® inverted microscope (Olympus, Center Valley, PA). Photos of whole alginate gels were taken with a Nikon COOLPIX® p90 camera (Nikon, Melville, NY).

2.13. Graphs and statistical analysis

Flow cytometry data were analyzed and plotted using FlowJo® software (Tree Star, Ashland, OR), and all other graphs were made using Kaleidagraph® software (Synergy Software, Reading PA). Statistical analysis was performed using Microsoft® Excel (Microsoft, Redmond, WA) or Kaleidagraph® software. A two-tailed Student's *t*-test assuming equal variances was used when comparing two groups, and a one-way analysis of variance (ANOVA) followed by a post hoc Tukey test was used when comparing multiple groups. For all experiments $n = 3–4$ unless otherwise indicated. Data are reported as the mean ± standard deviation.

3. Results

3.1. Endotoxin testing

All polymer solutions, calcium crosslinkers and other ion-supplemented solutions used in this study tested ≤0.1 EU ml^{-1}.

3.2. DC Comparison across materials

To determine if Ca^{2+}-crosslinked alginate gels had a distinct effect on DC behavior, DCs encapsulated in 40 mM $CaSO_4$ alginate gels were compared to DCs encapsulated in agarose and collagen gels and DCs plated on TCPS. Agarose was chosen because, like alginate, it is a polysaccharide that lacks integrin binding motifs and cannot be enzymatically degraded by mammalian cells; collagen and TCPS were chosen as standard biomaterial controls. In order to cast DCs in alginate gels at the bottom of wells consistently with collagen and agarose gels, $CaSO_4$ was used as the crosslinker because it is less soluble in aqueous solution than $CaCl_2$ (a more commonly used calcium crosslinker), leading to a slower gelation rate and the ability to mold alginate gels into the bottom of wells. DCs in alginate or agarose gels had a rounded appearance throughout the duration of the experiment, while cells on TCPS and in collagen were able to migrate, spread and make direct cell–cell contacts (Fig. 1A).

To determine if there were differences in DC activation across the materials, cell culture supernatants were bioplexed for various cytokines. DCs encapsulated in calcium-crosslinked alginate gels produced significantly higher levels of the inflammatory cytokine IL-1β when compared to DCs encapsulated in agarose and collagen gels and DCs plated on TCPS (Fig. 1B). Overall cytokine expression increased for all conditions when cells were stimulated with LPS, but IL-1β was even further enhanced with alginate gels (Fig. 1B). Interestingly, with LPS stimulation, DCs in agarose gels produced significantly higher levels of TNF-α, while DCs plated on TCPS produced significantly higher levels of IL-6. IL-4 and IFN-γ secretion were negligible for both LPS-free and LPS stimulated cells in all conditions and thus excluded from the rest of the study.

The impact of each gel on Ca^{2+} levels was next analyzed, since it is known that in physiological buffers, such as PBS, cell culture medium or serum, monovalent cations (e.g. Na^{+}) compete with divalent cations crosslinking the alginate, causing the divalent cations to be released over time [23]. Over a 10 h period, Ca^{2+}-cross-linked alginate gels increased the $[Ca^{2+}]$ in the surrounding medium to ~5 mM (indicating that ~43% of the calcium initially incorporated into the gel was released), while the other gels did not impact the Ca^{2+} concentration (Fig. 1C).

3.3. Testing polymer components of gels on TCPS-cultured DCs

The impact of the polymer components of the three hydrogels tested were next examined by culturing DCs on TCPS overnight with each of the three dissolved polymers. In the absence of LPS, IL-6 secretion for alginate was slightly higher than the no polymer (NP) condition

(Fig. 2), and in the presence of LPS, IL-1β and IL-12p70 secretion for collagen and agarose, respectively, were slightly different from NP (Fig. 2). However, these differences were minor and did not account for the trends seen with intact gels. In addition to the polymers used to fabricate gels, various alginate polymers of different molecular weight and M and G ratio (Supplementary Table S.1) were also screened and did not induce or enhance cytokine expres-

3.4. Testing Ca^{2+}-supplemented medium on TCPS-cultured DCs

The impact of free Ca^{2+} on cytokine secretion was tested next. DCs were cultured on TCPS in medium supplemented with Ca^{2+} so that the final $[Ca^{2+}]$ equaled 3, 6 or 12 mM. Interestingly, DCs extended longer processes (indicative of activation) as $[Ca^{2+}]$ in the medium increased, particularly for 12 mM (Fig. 3A).

Fluo-4, a fluorescent intracellular calcium probe, was used to quantify the levels of $[Ca^{2+}]_i$ for each of the conditions. Both photomicrographs (Supplementary Fig. S.2) and microplate readings (Fig. 3B) revealed that the $[Ca^{2+}]_i$ levels rose with increasing extracellular $[Ca^{2+}]$. Although $[Ca^{2+}]_i$ were highest immediately after plating and fluctuated within the first hour, a sustained high $[Ca^{2+}]_i$ was observed and maintained for at least 10 h (Fig. 3B).

Despite the fact that DCs in the 3, 6 and 12 mM $[Ca^{2+}]$ conditions looked equally as viable as the 0.42 mM conditions, forward-and side-scatter analysis using flow cytometry revealed that high $[Ca^{2+}]$ reduced cell viability in a dose-dependent manner and that LPS stimulation further reduced cell viability (Fig. 3C). The live/ dead populations gated using forward- and side-scatter were confirmed to be alive or dead using a live/dead fixable dye (Supplementary Fig. S.3).

Bioplex of cell culture supernatants showed that high $[Ca^{2+}]$ led to an increase in IL-1β both with and without LPS stimulation (with secretion peaking at 3 mM calcium) (Fig. 3D). These data were consistent with DCs cultured in calcium alginate gels. Interestingly, in the LPS-free condition, high extracellular $[Ca^{2+}]$ also upregulated IL-6 and TNF-α secretion, but in the presence of LPS, had no effect on IL-6 and actually downregulated IL-12p70 and TNF-α (Fig. 3D). To confirm that these trends were due to excess Ca^{2+} enhancing Ca^{2+} signaling and not an artifact of the ion, the medium was supplemented with high concentrations of other physiologically relevant ions (Mg^{2+}, Na^+ or K^+) or the Ca^{2+} ionophore A23187, a small molecule that increases $[Ca^{2+}]_i$ without changing the total $[Ca^{2+}]$ in the cell's environment, and after 24 h, supernatants were bioplexed for cytokines. The increase in IL-1β, IL-6 and TNF-α observed with calcium was repeated only with the Ca^{2+} ionophore A23187 (Supplementary Fig. S.4A). Additionally, an IL-1β control (86–144 pg ml^{-1}) was reconstituted in deionized H_2O or deionized H_2O supplemented with 12 mM Ca^{2+}, and no difference in concentration was detected by ELISA (Supplementary Fig. S.4B).

DCs cultured in increasing $[Ca^{2+}]$ were stained with anti-CD86 and anti-MHC class II antibodies, and live cells were gated into populations of negative, low or high expression of each marker based on flow cytometry histograms (Supplementary Fig. S.5). In LPS-free medium, increasing extracellular $[Ca^{2+}]$ correlated with increasing MHC class $II^{hi}CD86^{hi}$ double-positive expression in a dose-dependent manner, while LPS stimulation caused MHC class $II^{hi}CD86^{hi}$ double-positive cells to plateau at ~80% for all conditions (Fig. 3E).

3.5. Testing Ba^{2+} and Ca^{2+} crosslinker in alginate gels

Since the data indicated that it was the calcium crosslinker and not the alginate polymer accounting for enhanced DC maturation, we hypothesized that DCs encapsulated in Ca^{2+}-crosslinked alginate would have a greater degree of maturation than DCs encapsulated in Ba^{2+}-crosslinked alginate. To test this, DCs were encapsulated in alginate beads crosslinked

in a 10 mM $BaCl_2$ or 100 mM $CaCl_2$ bath, which is the most standard method for cross-linking alginate, and analyzed for their ability to induce DC maturation. 100 mM $CaCl_2$ was chosen because it is a concentration typically used for biomedical applications, and 10 mM $BaCl_2$ was selected because it yielded beads approximately the same size as 100 mM $CaCl_2$. As hypothesized, DCs encapsulated in Ca^{2+}-cross-linked alginate secreted significantly more IL-1β compared to DCs in Ba^{2+}-crosslinked gels as detected by ELISA (Fig. 4A). LPS stimulation caused an overall increase in IL-1β, but the presence of Ca^{2+} even further enhanced its secretion. Likewise, for both LPS-free and LPS conditions, DCs extracted from Ca^{2+}-crosslinked alginate gels had increased expression of CD86 and MHC class II compared to DCs extracted from Ba^{2+}-crosslinked alginate gels (Fig. 4B).

Since the calcium crosslinker was responsible for the enhanced IL-1β secretion and maturation marker expression observed with alginate gels, we then hypothesized that DCs encapsulated in alginate gels with increasing concentrations of calcium crosslinker would have a dose-dependent response. We found that increasing the $CaCl_2$ crosslinking concentration in alginate beads from 10 to 100 mM raised the $[Ca^{2+}]$ of surrounding medium, increased $[Ca^{2+}]_i$, decreased cell viability, significantly enhanced IL-1β secretion, and increased the expression of MHC class II and CD86 (Supplementary Fig. S.6), which was consistent with results seen with two-dimensional plated DCs cultured in increasing $[Ca^{2+}]$ (Section 3.4). To confirm that these results were not unique to $CaCl_2$-crosslinked alginate beads, we tested alginate discs cured with 10 or 40 mM $CaSO_4$, and the same trends were observed (Supplementary Fig. S.7).

To establish that DCs cultured externally from gels could also be matured by the Ca^{2+} released from calcium alginate gels, DCs were plated on TCPS in the absence or presence of alginate gels cross-linked with 4 mM $BaCl_2$ or 48.8 mM $CaSO_4$. These crosslinkers and crosslinking concentrations were chosen because they yielded gels that could be injected for *in vivo* testing. Similar to results seen above, DCs cultured in the presence of calcium alginate gels secreted significantly higher concentrations of IL-1β (Fig. 5A) and expressed higher levels of CD86 and MHC class II (Fig. 5B) compared to control and barium alginate conditions.

3.6. In vivo studies

To determine if calcium alginate gels could enhance inflammatory responses as demonstrated *in vitro*, 50 μl alginate gels cross-linked with 4 mM $BaCl_2$ or 48.8 mM $CaSO_4$, with or without 1 μg LPS, were injected medially into mice. Because alginate gels were injected into the body, which is an open system where Ca^{2+} can diffuse freely in and out, we were highly interested in analyzing the $[Ca^{2+}]$ within the gels over time. More than 50% of the calcium originally incorporated within calcium alginate gels was released within the first 12 h (Fig. 6A), and the $[Ca^{2+}]$ within the gels was maintained at a steady state thereafter. Interestingly, the $[Ca^{2+}]$ in barium alginate gels increased over time and also reached steady state by ~12 h. Thus, by the time gels were analyzed at 24 h, $[Ca^{2+}]$ in both gels were identical.

Although the $[Ca^{2+}]$ in both gels were identical at the time of analysis, calcium alginate gels induced more IL-1β secretion compared to barium alginate gels, and LPS delivered from calcium alginate gels more than quadrupled IL-1β secretion relative to LPS delivered from barium alginate gels (Fig. 6B). Despite most of the Ca^{2+} being released within the first 12 h, IL-1β could be detected within the gels for at least 4 days (Supplementary Fig. S.8). To get a more comprehensive analysis of other inflammatory mediators that calcium alginate gels induced from surrounding tissue, gels were multiplexed for 23 various cytokines and chemokines. We found that chemokines (MCP-1, MIP-1β, eotaxin, RANTES, MIP-1α and KC) were generally secreted in higher concentrations, and LPS delivered from calcium gels

upregulated MIP-1β, RANTES, MIP-1α, IL-1β, and IL-1α by ~3–5-fold greater than LPS delivered from barium gels (Fig. 6C). These data were consistent with *in vitro* data.

4. Discussion

The results of this study indicate that the Ca^{2+} used to crosslink alginate gels was released over time and promoted IL-1β, CD86 and MHC class II expression by DCs, whether they were encapsulated within gels or cultured externally from gels *in vitro*. In addition to increasing DC maturation, increasing extracellular [Ca^{2+}] was also associated with increasing $[Ca^{2+}]_i$ and reduced cell viability. Consistent with *in vitro* results, Ca^{2+} crosslinked alginate gels significantly promoted inflammatory cytokine secretion and enhanced the effects of LPS *in vivo*.

Contrary to published data showing that alginate polysaccharides could stimulate inflammatory cytokine production from monocyte populations, the various soluble alginate polysaccharides used in these experiments did not stimulate bone-marrow-derived DCs. Alginate has been suggested to activate monocytes and macrophages, potentially via the NF-κβ pathway [18,19,24–26], and several studies have reported the detection of antibodies against alginate *in vivo* [27–29]. Conflicting with this, alginate has been used as an anti-inflammatory to suppress experimental glomerulonephritis and ulcerative colitis [20,30]. The literature on alginate immunogenicity is abundant yet controversial, and many of these results must be interpreted with caution as alginates used in past studies may have contained impurities such as endotoxins, residual proteins or polyphenols, all of which affect the immunogenicity of alginate [21,31–33]. The alginate used in this study was of ultrapure grade, which may explain why no immunostimulatory effects were seen.

Although agarose polymers did not induce or affect DC activation, it was observed that whole agarose gels had a unique effect on DC activation. The gels themselves did not induce cytokine secretion from DCs but greatly enhanced TNF-α production when DCs were pulsed with LPS. It has been shown that heat shock proteins (HSPs) play an important role as molecular chaperones of the LPS-signaling pathway [34,35], and it is possible that the heated agarose, although cooled near body temperature before use, elicited the production of HSPs by DCs, subsequently enhancing TNF-α secretion when the cells were exposed to LPS. Although further testing would have to be done to confirm this, it is an interesting idea that could be used advantageously to activate white blood cells.

Calcium alginate gels increased the [Ca^{2+}] in the medium up to 5 mM (2.5 times what is physiologically relevant) over a 10 h period, and Fluo-4 labeling revealed that the elevated level of extracellular [Ca^{2+}] whether it be on TCPS or with alginate gels, resulted in a sustained increase in $[Ca^{2+}]_i$. The exact mechanism(s) of increased $[Ca^{2+}]_i$ in this study was not determined, but it was likely due to a series of events leading to contributions from both intracellular Ca^{2+} stores and extracellular Ca^{2+} [36,37]. Given the intricately intertwined activities of ion channels, pumps, transporters, intracellular buffers, Ca^{2+}-binding proteins and organelles, all of which affect $[Ca^{2+}]_i$ [37], one would have to use more sophisticated techniques, such as patch clamp techniques, to precisely determine the mechanisms of increased $[Ca^{2+}]_i$ in these experiments.

A critical finding of this study was that increasing [Ca^{2+}] matured DCs and enhanced LPS-induced DC activation. Dendritic cells and other leukocytes are highly dependent on Ca^{2+} to carry out their effector functions, and several intracellular Ca^{2+}-sensing and -signaling molecules have been identified as having necessary roles in their activation [3–5,8]. It is likely that the increases in $[Ca^{2+}]_i$ induced by raising extracellular [Ca^{2+}] activated these Ca^{2+} signaling molecules, leading to the upregulation of DC activation marker expression

and inflammatory cytokine secretion. These results illustrate how one can amplify protein expression by increasing a second messenger (in this case Ca^{2+}) that is downstream of a signaling event (such as TLR signaling). It is also plausible that the Ca^{2+} being released from the alginate gels was sensed externally by the G-protein-coupled extracellular Ca^{2+}-sensing receptor, CaR, initiating various signaling pathways leading to expression of inflammatory markers [38].

One of the most striking findings was the increase in IL-1β observed with calcium alginate gels. IL-1β is a critical mediator of inflammation, with important roles in neutrophil mobilization, cellular adhesion to the endothelium and white blood cell infiltration [39,40]. Cleavage of pro-IL-1β into its functional, secreted form is predominantly mediated by the NRLP3 inflammasome, an important molecular platform expressed by myeloid cells in innate immune defense that can be activated by a number of danger signals and stress factors [41]. A connection has been made between Ca^{2+}-induced mitochondrial damage and activation of the NLRP3 inflammasome, which likely explains the increase in IL-1β [41–43]. Inflammasome activation has been implicated in the success of adjuvants, such as alum [44,45], which suggests that Ca^{2+} induction of IL-1β (and potentially inflammasome activation) may have interesting implications in the field of vaccination.

The $[Ca^{2+}]$ levels in this study correlated with reduced cell viability in a dose-dependent manner. This is consistent with past reports showing that elevations in $[Ca^{2+}]_i$ can directly and indirectly induce cell injury and death [2]. The largest contributor to Ca^{2+} toxicity is believed to be Ca^{2+}-induced mitochondrial permeability transition (MPT), which is the formation of a large pore in the mitochondrial membrane during mitochondrial stress [2]. When cytosolic Ca^{2+} is elevated (>500 nM), mitochondria can be destabilized, initiating MPT, and resulting in the release of pro-apoptotic proteins. Cell death due to Ca^{2+} toxicity may have accounted for decreases in IL-1β seen with TCPS for $[Ca^{2+}]$ above 3 mM. Additionally, LPS-induced TNF-α secretion could have triggered apoptosis via the TNF pathway and accounted for the decreased viability seen with LPS-treated conditions [46].

When calcium alginate gels were injected *in vivo*, it was demonstrated that this could induce inflammatory cytokine secretion from surrounding tissue. Similar to Ca^{2+} release profiles *in vitro*, the majority of the Ca^{2+} was released within the first few hours. Interestingly, even though $[Ca^{2+}]$ in both calcium and barium alginate gels equilibrated within the first 12 h, we were still able to detect strong differences in IL-1β between the two gels at the time of analysis. These bioplex results, along with our bioplex data showing that only A23187 was able to reproduce the effects of supplementary Ca^{2+} and our ELISA data showing that antibody binding to IL-1β was not Ca^{2+}-dependent, are strong evidence that the phenomena observed were due to Ca^{2+} signaling enhancing cytokine secretion and not due to a change in osmolality or an artifact of excess Ca^{2+} influencing antibody detection of the cytokines. Since IL-1β could be detected within alginate gels beyond the first day, it is possible that the gels sequestered cytokines induced by the burst release of Ca^{2+}, but slowly released these cytokines as inflammation subsided. Consistent with results seen *in vitro*, Ca^{2+} and LPS released from alginate gels had a synergistic effect and activated the most IL-1β secretion observed *in vivo*. Aside from IL-1β, strong upregulations in other inflammatory cytokines and chemokines were also observed.

Although the majority of studies utilizing calcium alginate gels for biomedicalpurposes have not examined the effects of the calcium crosslinker, evidence in some studies is consistent with our findings. When activated dendritic cells were delivered in Ca^{2+}-crosslinked alginate gels to reduce tumor size in mice, calcium alginate gels alone seemed to have a slight therapeutic effect. Consequently, it would be interesting to see if replacing the Ca^{2+} with Ba^{2+} would reduce vaccine efficacy [16]. Also mentioned earlier was that Ca^{2+}-

crosslinked alginate gels had a positive effect on angiogenesis in a mouse hindlimb ischemia model [15]. Although not statistically significant, alginate alone appeared to increase blood vessel density over untreated animals and animals injected with a bolus dose of vascular endothelial growth factor (VEGF). It is possible that the Ca^{2+} in the gels promoted the secretion of endogenous pro-angiogenic factors from immune and/or non-immune cells, leading to enhanced blood vessel formation. Lastly, alginate has been used for decades in the management of acute and chronic wounds, although its exact molecular and cellular effects are not well-understood. There has been a recent report that alginate promoted keratinocyte differentiation, which is critical for wound healing, and this was due to the Ca^{2+} released by the alginate [47]. These examples illustrate the importance of characterizing the effects of Ca^{2+} on other leukocytes and non-immune cells both *in vitro* and *in vivo*.

5. Conclusion

We found that the Ca^{2+} released from calcium alginate gels led to DC maturation and enhanced LPS-induced inflammatory cytokine secretion *in vitro*. Likewise, calcium alginate cells injected subcutaneously in mice were able to upregulate a number of inflammatory cytokines and chemokines relative to barium alginate, and the inflammatory effects of LPS on surrounding tissue were enhanced when LPS was delivered from calcium alginate vs. barium alginate. This work suggests that it is important to take into consideration the effects of calcium crosslinker when using alginate gels for biomedical applications and may have significant implications in future biomaterial design.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We would like to thank Beth Ann Lopez (University of New Mexico – Albuquerque) for her help with preliminary experiments and Dr. Kamal Bouhadir (American University at Beirut) for generously providing purified M and G block alginate polymers. This work was supported by the NIH (R01DE019917).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.actbio.2013.08.002.

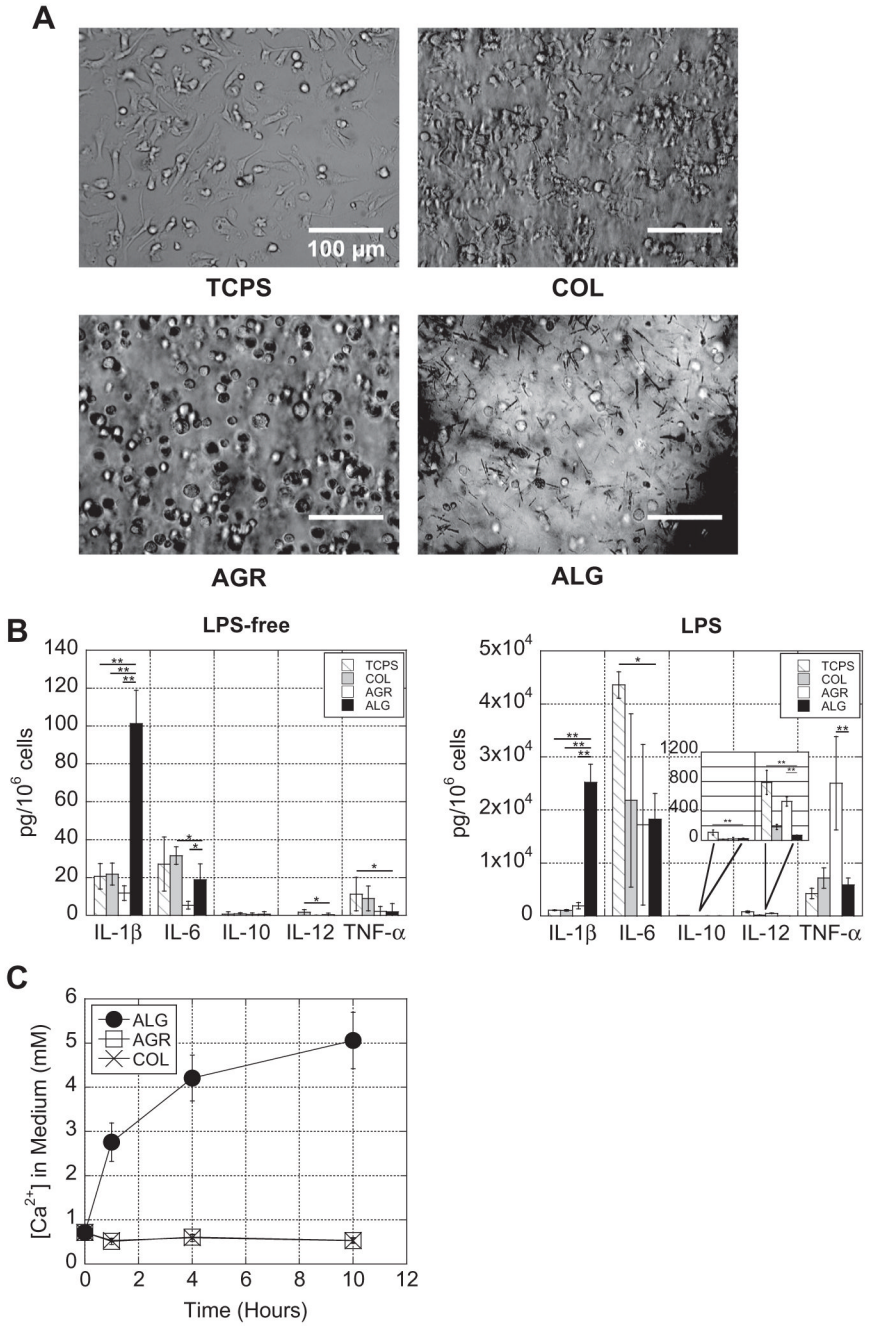

Fig. 1.

Alginate gels enhanced IL-1β secretion from encapsulated DCs. (A) Photomicrographs were taken of DCs adhered to TCPS or encapsulated in collagen (COL), agarose (AGR) or alginate (ALG) gels 1 h after encapsulation. (B) DCs were cultured without LPS or with LPS. After 24 h, supernatant was collected and analyzed for multiple cytokines. (C) The Ca^{2+} released into medium from each of the hydrogels was quantified over 10 h. $^{*}P \leq 0.05$; $^{**}P \leq 0.001$.

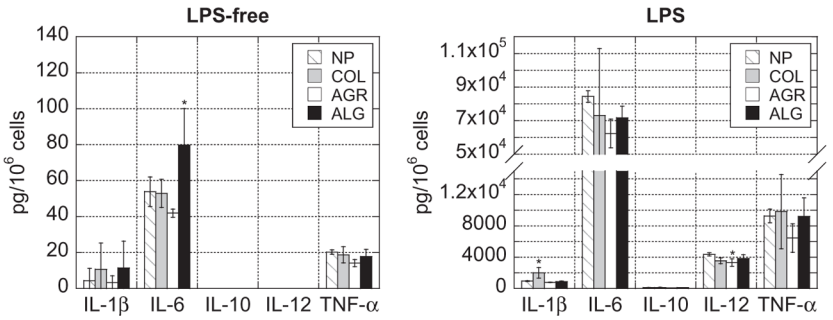

Fig. 2.

Soluble polymer components of gels were not responsible for trends seen with intact gels. DCs were cultured on TCPS with no polymer (NP), or dissolved collagen (COL), agarose (AGR) or alginate (ALG), in the absence of LPS or in the presence of LPS. After 24 h, supernatant was collected and multiplexed for cytokines. Asterisks indicate that the condition is significantly different from the NP condition. *P<0.05.

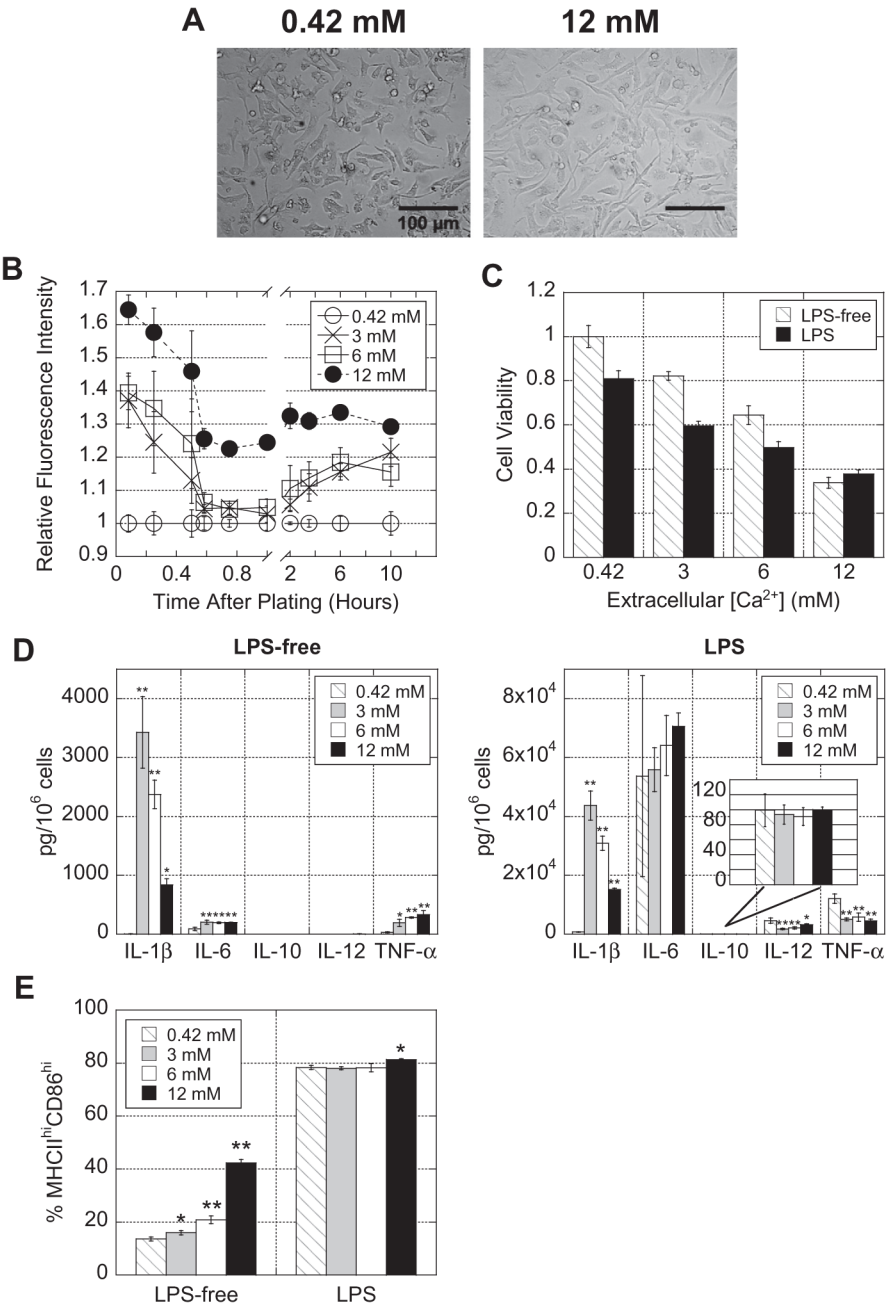

Fig. 3.

Ca^{2+} promotes DC maturation. DCs were plated in increasing [Ca^{2+}] in the absence or presence of LPS for 24 h. (A) Photomicrographs were taken of DCs (only 0.42 and 12 mM are shown). (B) DCs were labeled with the intracellular Ca^{2+} probe Fluo-4 and fluorescence was quantified over a 10 h period using a plate reader. (C) Cells were collected and viability was quantified based on forward-/side-scatter measurements obtained using flow cytometry. (D) Supernatants were multiplexed for cytokines. (E) DCs cultured in increasing [Ca^{2+}] were stained with PE-labeled anti-CD86 and FITC-labeled anti-MHC class II and analyzed using flow cytometry. Asterisks indicate that the Ca^{2+}-supplemented condition is significantly different from the basal (0.42 mM) condition. $^{*}P \leq 0.05$; $^{**}P \leq 0.001$.

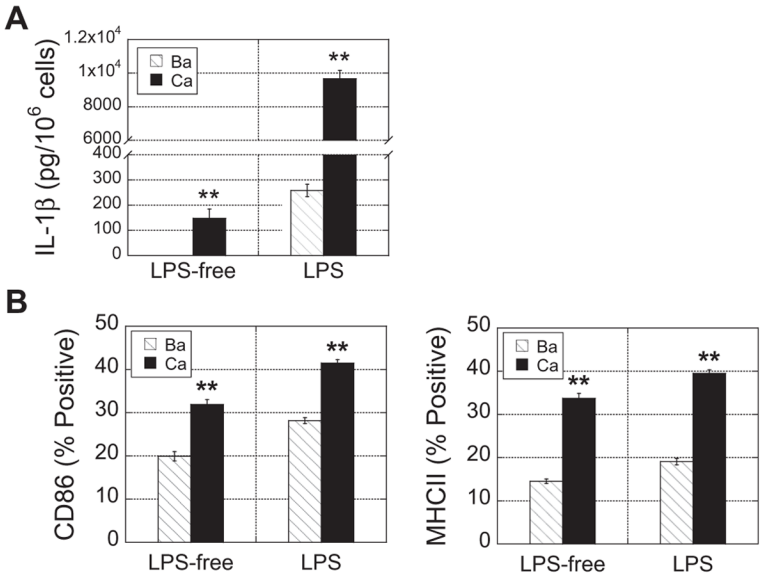

Fig. 4.

Calcium crosslinker enhanced activation markers of encapsulated DCs compared to barium crosslinker. (A) After 24 h in culture, supernatants from DCs encapsulated in barium (10 mM) or calcium (100 mM) alginate beads, in the absence or presence of LPS, were collected and assayed for IL-1β. (B) DCs were extracted from beads and analyzed for CD86 and MHC class II using flow cytometry. Asterisks indicate that the calcium condition is significantly different from the barium condition. $^{**}P \leq 0.001$.

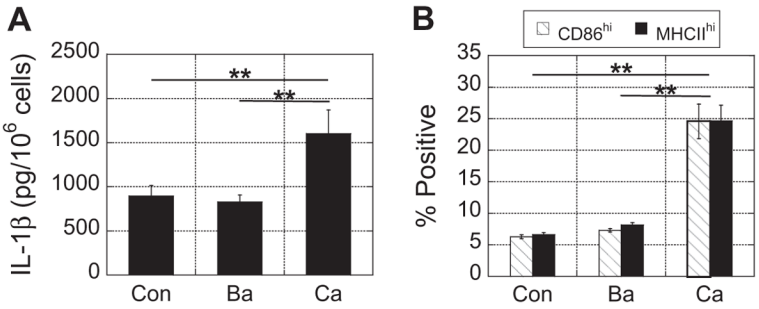

Fig. 5.

Calcium crosslinker enhanced activation markers of DCs cultured outside of gels compared to barium crosslinker. (A) After 24 h in culture, supernatants from DCs plated on TCPS and cultured in the presence of barium (4 mM) or calcium (48.8 mM) alginate gels were collected and assayed for IL-1β. (B) DCs were scraped from wells and analyzed for CD86 and MHC class II using flow cytometry. $^{**}P \leq 0.001$.

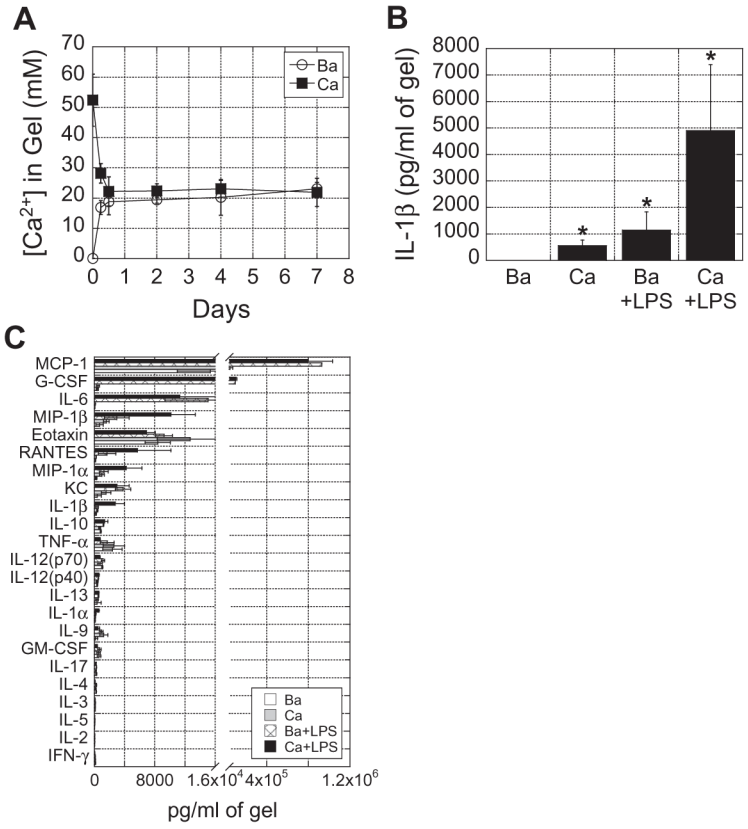

Fig. 6.

Calcium alginate gels induce greater inflammatory cytokine secretion compared to barium alginate gels and enhanced LPS-induced inflammation *in vivo*. (A) 50 μl of barium (4 mM) and calcium (48.8 mM) alginate gels were injected into subcutaneous tissue of mice. At various timepoints, alginate gels were removed and analyzed for $[Ca^{2+}]$. (B) 50 μl of barium and calcium alginate gels, with or without LPS, were injected into mice. After 24 h, gels were harvested and analyzed for IL-1β concentration and (C) other inflammatory cytokines and chemokines. Asterisks indicate that the condition is significantly different from the barium only condition. $^{*}P < 0.05$.