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Controlled Release of Stem Cell Secretome Attenuates Inflammatory Response against Implanted Biomaterials

Mohammadreza Mohammadi, Jennifer Cam Luong, Samuel Mathew Rodriguez, Rui Cao, Ashlyn Elizabeth Wheeler, Hien Lau, Shiri Li, Sepideh Kiani Shabestari, Jean Paul Chadarevian, Michael Alexander, Paul de Vos, Weian Zhao, and Jonathan Robert Tod Lakey*

Inflammatory response against implanted biomaterials impairs their functional integration and induces medical complications in the host's body. To suppress such immune responses, one approach is the administration of multiple drugs to halt inflammatory pathways. This challenges patient's adherence and can cause additional complications such as infection. Alternatively, biologics that regulate multiple inflammatory pathways are attractive agents in addressing the implants immune complications. Secretome of mesenchymal stromal cells (MSCs) is a multipotent biologic, regulating the homeostasis of lymphocytes and leukocytes. Here, it is reported that alginate microcapsules loaded with processed conditioned media (pCM-Alg) reduces the infiltration and/or expression of CD68+ macrophages likely through the controlled release of pCM. In vitro cultures revealed that alginate can dose dependently induce macrophages to secrete TNF α , IL-6, IL-1 β , and GM-CSF. Addition of pCM to the cultures attenuates the secretion of TNF α ($p = 0.023$) and IL-6 ($p < 0.0001$) by alginate or lipopolysaccharide (LPS) stimulations. Mechanistically, pCM suppressed the Nf κ B pathway activation of macrophages in response to LPS ($p < 0.0001$) in vitro and cathepsin activity ($p = 0.005$) in response to alginate in vivo. These observations suggest the efficacy of using MSC-derived secretome to prevent or delay the host rejection of implants.

Long-term biocompatibility and function of implanted scaffolds require absence of detrimental immune response.^[1] The immune response that is also known as foreign body response (FBR) could be divided into sequential steps of tissue injury, immunocytes recruitment and adhesion, induction of myofibroblasts, and fibrotic capsule formation. Such events prevent the interaction of the implants with the surrounding microenvironment, leading to the non-functionality of the implants and complications in the host's body. A few examples that FBR creates issues for the implants are signal inaccuracy in glucose measurements of continuous glucose monitors,^[2] graft rejections of encapsulated pancreatic islets,^[3] and complications of breast implants that recently led FDA to recall BIOCELL textured breast implants from market.^[4] To alleviate such immune and fibrotic responses, multiple approaches have been described previously, including manipulation of size,^[3a,5] shape,^[6] surface characteristic,^[7] and fabrication of devices with controlled release of anti-inflammatory agents.^[3b,8]

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Inflammatory response against implanted biomaterials depend upon the structural and physiochemical properties of the material, where multiple signaling pathways are involved.^[9] One example of FBR is the fibrotic cascade against implanted alginate microcapsules containing pancreatic islets for the glycemic control in Type 1 diabetics. The onset of FBR is upon surgical procedure prior to transplantation, leading to neutrophils activation and migration.^[10] Inflammatory response further proceeds with the activation and recruitment of macrophages, B cells and T cells around microcapsules.^[3a,9a,10b,11] Within weeks to months, immunocytes populate the surface of microcapsules, interfering the accessibility of insulin outward and of nutrients inward the microcapsules. In the long term, implants get encapsulated and fibrosed.

The result of this immune myriad is the non-functionality of the transplant known as graft failure. Thus, strategies have been developed to regulate the inflammatory response against alginate microcapsules for long-term efficacy of pancreatic islet transplantation.^[8,12] Among different immunomodulatory approaches, alginate's surface engineering^[12-13] and loading of alginate with immunomodulatory factors^[8] have been extensively described previously. It is therefore expected that a controlled-release biomaterial with releasing multi-potent anti-inflammatory agent may better shield the implants against the host immune interactions to halt fibrotic cascade.

In this context, mesenchymal stromal cells (MSCs) are known as multipotent cells that regulate variety of inflammatory pathways including NF- κ B,^[14] JAK/STAT,^[15] MyD88,^[16] PI3K/AKT,^[17] and PGE2 downstream pathways.^[18] MSCs are under investigation in more than 700 clinical trials, mostly focused on inflammatory and autoimmune disorders. While their therapeutic mechanisms are remained to be fully elucidated, MSCs are suggested to exert their immunomodulatory characteristics partly through paracrine factors.^[17,19] Cumulated body of evidence suggests that secretome of MSCs has immunosuppressive and immunoregulatory potentials, interacting with different classes of immunocytes.^[14,19b,d] We thus prepared alginate microcapsules loaded with processed condition media (pCM) secreted from human Umbilical Cord Derived MSCs (pCM-Alg) and subcutaneously implanted into C57/BL6 (immunocompetent) mice. After 2 weeks, we removed and studied the explants of pCM-Alg as well as Ctrl-Alg microcapsules. We hypothesized that pCM will be released into the host's microenvironment and dampens the inflammatory response against microcapsules (**Figure 1a,b**).

To observe the difference in immune-profiles, we collected the whole-mount explants and stained for total cell infiltration as well as two FBR markers, that is, CD68+ (macrophages) and α SMA+ (alpha smooth muscle actin) cells (**Figure 1c**). To compare the number of cells, signal area was measured in similar magnifications and normalized by the total tissue area in the image.

Figure 1d shows the quantification for the DAPI area, which indicates the reduction in total cell infiltration ($n = 4$, $p = 0.015$) around pCM-Alg (normalized area of DAPI % = $4.54\% \pm 2.25\%$, mean $\pm$ SD) compared to control (normalized area of DAPI% = $12.26\% \pm 3.64\%$). Normalized area of CD68+ macrophage also reduced from $4.86\% \pm 1.81\%$ to $0.94\% \pm 0.47\%$ ($n = 4$, $p = 0.019$) in control and pCM-Alg (**Figure 1d**). No difference in the α SMA+ cells between two groups were found ($n = 4$, $p = 0.99$; normalized area % = $3.01\% \pm 1.38\%$ and $4.19\% \pm 3.3\%$ for Ctrl-Alg and pCM-Alg, respectively).

We further sought to characterize and quantify the immune-profiles for both pCM-Alg and Ctrl-Alg microcapsules. We thus explanted microcapsules after 2 weeks implantation and sectioned the microcapsules for immunostaining. **Figure 1e** shows the images of fibrotic tissue captured prior to explantation, which were then micro-sectioned into 5–10 μ m slices. We then stained the slices with H&E (**Figure 1f**) and conducted immunofluorescence (**Figure 1g**) to visualize the inflammatory milieu around microcapsules. These results suggest that, at least 2 weeks after implantation, total cell infiltration and CD68+ around pCM-Alg were lower than Ctrl-Alg. To further quantify cell infiltration around implants, we DAPI stained fibrotic tissues and counted each DAPI+ area as an individual cell using IMARIS software. Total cell infiltration around pCM-Alg was significantly lower ($p = 0.005$) around the pCM-Alg (722 ± 101) microcapsules compared to Ctrl-Alg (1562 ± 772) after 2 weeks of implantation (**Figure 1h**).

After the initial inflammatory response against the implants, fibroblasts get recruited around the inflammatory microenvironment. Fibroblasts are then responsible for final fibrous collagen and matrix protein deposition.^[9a] We therefore sought to quantify the collagen quantity around the microcapsules. **Figure 1i** demonstrates the Masson's Trichrome staining and quantification of collagen (blue staining). Using the imageJ software to quantify collagen (as described in Supporting Information), we found that there is significantly less collagen around the pCM-Alg microcapsules compared to CTRL-Alg ones ($p = 0.0034$). This result further suggests the less-fibrotic response around pCM-Alg microcapsules.

These results offer that, at least during the first 2 weeks after implantation, pCM-Alg alleviates the initial inflammatory response, whereby fewer total cells and CD68+ macrophages are infiltrated around alginate microcapsules. We further hypothesized that reduction of cell infiltration could be partly explained via the release of pCM to the surrounding microenvironment of microcapsules. Upon release, pCM dampens the local inflammation likely through interacting with infiltrating/adjacent immunocytes. To measure the release profile, we first quantified the protein amount encapsulated in pCM-Alg microcapsules and found that ≈ 1000 pCM-Alg microcapsules contain $901.6 \pm 143.9 \mu\text{g mL}^{-1}$ proteins. The encapsulated protein amount reduced to $197 \pm 88.3 \mu\text{g mL}^{-1}$ after 48 h of incubation at 37 °C (**Figure 2a**), suggesting the overtime reduction of protein within pCM-Alg microcapsules. To speculate the protein release profile, we collected 0.5 mL of the media around pCM-Alg microcapsule and measured the protein content. **Figure 2b** shows the cumulative protein release, demonstrating the controlled release profile from pCM-Alg over 48 h (green line graph). These results in their totality suggest that pCM-Alg releases its protein content overtime.

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Figure 1. Cell infiltration around pCM-Alg is less than control microcapsules. Both control and pCM microcapsules were implanted subcutaneously in C57/BL6 mice. 2 weeks after implantation, formed fibrotic tissues were extracted and analyzed for cell infiltration. Schematic representation of the reduction of inflammatory response in a) control alginates versus b) pCM-Alg microcapsules. c) Whole explants were analyzed for CD68 and α -SMA markers, and nuclei were counterstained with DAPI. Some capsules and magnified images (scale bar = 50 μ m) are marked for visual aid. e) Some of the microcapsules were imaged after 2 weeks of implantation and prior to explantation. Explants then fixed and sectioned into slices with 5 μ thickness. f) Tissue slides were stained with H&E and g) CD68 and α -SMA markers, and nuclei were counterstained with DAPI. Similar procedures and staining were also performed on microcapsules collected 4-weeks after implantation. h) Total cells infiltration was quantified using the counts of DAPI positive spots. i) Masson's Trichrome staining was used to quantify the collagen presence as a fibrotic marker, demonstrating the higher amount of collagen around CTRL-Alg microcapsules. In all experiments $n = 4$, and Unpaired t -test with Welch's correction was used to determine the statistical significance (n.s. non-significant; * $p < 0.05$).

Figure 2. pCM-Alg microcapsules release immunomodulatory MSCs secretome in vitro. **a)** protein content of freshly prepared pCM-Alg is $901.6 \pm 143.9 \mu\text{g mL}^{-1}$ per ≈ 1000 microcapsules, which is higher than that of CCM-Alg ($88.4 \pm 72.3 \mu\text{g mL}^{-1}$; $p < 0.001$). After 48 h of incubation, ≈ 1000 pCM-Alg microcapsules retain $197 \pm 88.3 \mu\text{g mL}^{-1}$ total protein, while CCM-Alg contains $27.6 \pm 23.7 \mu\text{g mL}^{-1}$ ($p = 0.003$). **b)** Total protein release for both pCM-Alg and CCM-Alg over 48 h, suggesting that proteins released from pCM-Alg are specific to MSC secretome rather than cell culture media. **c)** BCA assay was used to measure the protein amount in pCM and CCM, where pCM contains higher protein content ($1.22 \pm 0.10 \text{ mg mL}^{-1}$; $p = 0.02$) compared to culture media ($1.08 \pm 0.06 \text{ mg mL}^{-1}$). **d)** Proteins released from pCM-Alg at 8 h were collected and characterized for the EVs release. Nanoparticle tracking analyses (NTA) and **e)** Western blotting were used to count and verify the released EVs. In both cases, negative controls were proteins release from CCM-Alg at 8 h of incubation. **f)** To test the immunomodulatory effects of MSC secretome, peripheral human blood mononuclear cells (PBMCs) proliferation assay was conducted. PBMCs were activated by magnetic beads coated with anti-CD3 and anti-CD28 in the presence and absence of pCM for 4 days. Supernatants of cultures were isolated, and cytokines were analyzed. We found that addition of pCM to the activated PBMCs co-culture decreases the mean fluorescence intensity of IL-6 (twofold), IL-12p70 (4.7-fold), IFN γ (2.5-fold), TNF α (2.4-fold), and increases IDO (1.9-fold). Significance measurements were conducted by Unpaired *t*-test with Welch's correction.

The pCM (which is a processed conditioned media produced by MSCs) contains variety of proteins, which are coming from two separate sources. The first source is the proteins and growth factors in the cell culture media (CCM), and the second source is secreted factors from MSCs (secretome). In our previous experiments we demonstrated the release of proteins from pCM-Alg; however, we have not determined to what extent these proteins have originated from CCM or secretome. To gain a better understanding on the origin of the released proteins, we prepared a new alginate formulation that incorporates CCM into alginate microcapsules (CCM-Alg). We then attempted to measure the protein release from CCM-Alg and compare it against pCM-Alg. Figure 2a shows the total protein content for ≈ 1000 microcapsules of CCM-Alg ($88.4 \pm 72.3 \mu\text{g mL}^{-1}$) was significantly lower than that of pCM-Alg ($901.6 \pm 143.9 \mu\text{g mL}^{-1}$; $p < 0.001$). In a similar trend, Figure 2a shows that less proteins retained within CCM-Alg ($27.6 \pm 23.7 \mu\text{g mL}^{-1}$) compared to that of pCM-Alg ($197 \pm 88.3 \mu\text{g mL}^{-1}$; $p = 0.003$) after 48 h incubation. The $\approx$ tenfold more proteins encapsulated in pCM-Alg versus CCM-Alg could suggest two possibilities: In a similar volume, either pCM contains $\approx$ tenfold more proteins compared to CCM, or alginate could retain $\approx$ tenfold more proteins of pCM compared to

proteins of CCM. To investigate these possibilities, we first analyzed the protein amount in pCM and CCM. Figure 2c demonstrates the comparison between CCM and pCM, showing that pCM contains slightly higher amount of protein ($1.22 \pm 0.10 \text{ mg mL}^{-1}$; $p = 0.02$), compared to CCM ($1.08 \pm 0.06 \text{ mg mL}^{-1}$). Even though higher, the protein content within pCM does not justify a tenfold difference in the protein content of pCM-Alg and CCM-Alg. It should be noted that pCM was obtained from a 2-days of sera-free MSCs cultures, while CCM is obtained from 2-days of similar incubations but in the absence of MSCs.

We next sought to investigate the second possibility, which is hypothesizing that alginate could retain $\approx$ tenfold more pCM proteins compared to proteins of CCM. While many factors could affect more protein retention in pCM-Alg, we particularly hypothesized that proteins within pCM are larger than CCM proteins. Larger proteins then could not easily diffuse out of microcapsules, leading to higher protein retention in pCM-Alg microcapsules. It is now widely demonstrated that the secretome of MSC contains variety of extracellular vesicles (EVs),^[14,19b,d] and these vesicles are hybrid structures of lipid, proteins, and genetic materials. Indeed, it is well established that MSCs secrete variety of vesicles including apoptotic bodies,^[20] microvesicles,^[19f]

exosomes,^[17] lipoprotein particles,^[21] and ribonucleoproteins.^[22] Interestingly, these vesicles could recapitulate the therapeutic benefits of MSCs, stimulating research and development of cell-free therapy.^[17,23] The presence of these vesicles within the pCM-Alg (but not CCM-Alg) may partly explain the higher protein retention in pCM-Alg microcapsules.

We then focused our experiments to test whether EVs are entrapped within pCM-Alg and could be released from them. To test this possibility, we evaluated the released EVs from pCM-Alg after 8 h of incubation. To observe and quantify the released EVs from both pCM-Alg, and CCM-Alg, we conducted NTA analyses. Figure 2d shows the representative image of the vesicles captured via NTA, demonstrating the size of 107.6 ± 35.9 nm. No vesicle was observed in the released content of CCM-Alg (data not shown). To further characterize the visualized EVs, we conducted Western blotting analyses to identify some key biomarkers. Figure 2e shows the presence of widely-reported biomarkers TSG101,^[17,24] CD63,^[23a] and Galectin-1,^[17] in pCM-Alg released content, while they were absent in the CCM-Alg released proteins. We chose to represent 4 biomarkers, because based on minimal information for studies of extracellular vesicles (MISEV), presence of 3 biomarkers and absence of one putative contaminant are minimally required to characterize the EVs.^[25] Since cell debris could also contaminate the condition media, we also checked Calnexin, which is the endoplasmic reticulum contamination^[19f] in the released cargo, and it was not detectable in the released pCM and purified EVs (Figure 2e). In summary, released EVs have sizes matching the size range of exosomes,^[17,26] express exosomal biomarkers (negative for Calnexin and positive for TSG101,^[17,24] CD63,^[23a] and Galectin-1,^[17]). These results suggest that, at least part of the released EVs from pCM-Alg are likely to be exosomes secreted by MSCs. However, further experiments such as immunogold labeling and more extensive biomarkers are required to assure the presence and purity of exosomes. The heterogeneity of small EVs and presence of non-vesicular cargo have led to debate about contents and functional properties of exosomes.^[27]

Thus far, we observed that pCM-Alg reduces the FBR response in vivo and releases the MSC secretome in a controlled fashion. We then hypothesized that released secretome would modulate the immune-milieu around the pCM-Alg, leading to the reduced cell infiltration. Our hypothesis stems in the MSCs capability to regulate the immune response in part through secretion of cytokines and extracellular vesicles.^[14,17,19d] Similar concept has been developed in previous studies, where CXCL12^[3b,8b] and GW2580^[8a] were incorporated within alginate microcapsules to alleviate the FBR. To examine the immunomodulatory effects of pCM on immunocytes, we conducted human Peripheral Blood Mononuclear Cells (PBMCs) proliferation assay and isolated the supernatants to measure the cytokine secretion. PBMCs were activated by magnetic beads coated with anti-CD3 and anti-CD28, which mimics the antigen presentation to T lymphocytes. We found that addition of pCM to the activated PBMCs culture decreases the Mean Fluorescence Intensity of IL-6 (twofold), IL-12p70 (4.7-fold), IFN γ (2.5-fold), TNF α (2.4-fold), and increases IDO (1.9-fold), which are summarized in the Figure 2f. It is well documented that MSC can suppress PBMC proliferation in co-cultures mainly through secretory molecules including IDO,^[28] IL-10,^[29] PGE2,^[30] TSG6,^[31] and TGF β .^[32] Interestingly, the immunosuppressive effects of MSCs has been associated with their

secretory molecules,^[19b,c,33] further supporting our results from the PBMC proliferation cytokine panel.

Myriad of immune activation and immunocytes infiltration have been reported following by alginate microcapsules implantation, with macrophages as central players to orchestrate such responses.^[3a,9a,10b] Failure in the resolution of macrophage inflammatory response to the implants could lead to chronic inflammation and complications. We therefore focused our experiments to better understand the immunoregulatory of pCM on activated macrophages. Macrophages could be activated through variety of agonists and pathways. We particularly designed our in vitro macrophage activation experiments to simulate the immunostimulatory properties of alginates to better replicate the in vivo inflammatory conditions caused by alginate implantation.

Two mechanisms have been described to explain the reasons for alginate-based inflammatory response. Some studies have suggested the presence of immunogens within alginate (such as lipopolysaccharide (LPS), lipoteichoic acid, and peptidoglycans) are the main inducers of inflammation.^[34] Others reported that such contaminations were undetectable in their alginate,^[9a,10a,12] and linked the inflammatory response to the inherent properties of alginate. Alginate is a natural acidic polysaccharide extracted from marine brown seaweeds.^[9c,d] It is composed of different blocks of β -(1, 4)-D-mannuronate (M) and its C-5 epimer α -(1, 4)-L-guluronate (G), and guluronate oligosaccharide derived from alginate has been reported to readily activate macrophages partly through toll-like receptor 4 (TLR4) signaling pathway.^[9c,d] Either through presence of endotoxins or inherent ability of alginate to be immunogenic, TLR4 agonistic effect of alginate is one of the main shared immunostimulatory pathways among different reports. We therefore attempted to test whether pCM could resolve the immunogenicity of alginates and LPS against macrophages.

To test the immunosuppressive capabilities of pCM on myeloid lineage, we first tested whether pCM could inhibit the inflammatory response in both human- and murine-derived macrophages. We first attempted to test cytokine secretion from murine RAW 264.7 macrophages under stimulatory conditions. To stimulate macrophages, we used two doses of either alginate or LPS. Macrophages were stimulated with 0.125 or 1.250 mg mL⁻¹ of ultrapure UPLVG alginate for 12 h and secreted cytokines were collected for the analysis. Figure 3a shows the schematic representation of experimental procedure, where macrophages were incubated with 0.125 or 1.250 mg mL⁻¹ of UPLVG for 12 h in the presence or absence of pCM. We found that alginate significantly upregulates the secretion of TNF α and IL-6 in a dose dependent manner, and alginate also increases the production of IL-1 β , and GM-CSF, while not statistically significant (Figure 3b). Furthermore, addition of pCM significantly reduces TNF α and IL-6 secretion from alginate-stimulated RAW macrophages (Figure 3b).

To further validate the immunomodulatory effect of pCM on macrophages, we tested the level of CD68 marker on macrophages. CD68 upregulation is one of the biomarkers for macrophage activation in response to inflammatory stimuli,^[3d,35] including TLR4 stimulation.^[36] From the cultures, macrophages were then collected and analyzed for the CD68 expression (Figure 3c). Figure 3d demonstrates the quantification of CD68+ population, revealing that CD68 is upregulated via both doses of UPLVG stimulation. CD68 expression on native RAW macrophages

Figure 3. pCM reduces the inflammatory response in activated macrophages. a) Schematic representation of macrophages cultured with alginate in the presence and absence of pCM. b) Addition of 0.125 or 1.25 mg mL⁻¹ of alginate to the macrophages enhances the secretion of TNFα, IL-1β, and IL-6. Presence of pCM in the cultures significantly reduces the secretion of these cytokines. c,d) Alginate upregulates the CD68 expression of RAW macrophages, while addition of pCM to the cultures reduces CD68 expression. CD68 expression on native RAW macrophages was 1.41% ± 0.60%. CD68 expression increased to 5.89% ± 0.35% in the presence of 0.125 mg mL⁻¹, while addition of pCM reduced it to 2.71% ± 0.63% (*p* = 0.005). Addition of 1.25 mg mL⁻¹ alginate upregulated the CD68 expression to 7.64% ± 1.10%, while addition of pCM reduced it to 2.91% ± 1.20% (*p* = 0.008). e) Schematic representation of macrophages cultured with LPS in the presence and absence of pCM. f) Addition of 10 or 100 ng mL⁻¹ of LPS to the RAW macrophages upregulates the secretion of TNFα, IL-1β, IL-6, and GM-CSF, and addition of pCM significantly reduces these secretions. g,h) CD68 expression for the culture with 10 ng mL⁻¹ and 100 ng mL⁻¹ LPS was 10.96% ± 3.93% and 11.59% ± 2.75%, respectively. Addition of pCM to the culture reduced the CD68 expression to 4.53% ± 1.39% for the former (*p* = 0.0003), and 3.78% ± 1.29% for the later (*p* = 0.009). Statistical analysis was done by unpaired *t*-test analyses with Welch's correction.

was 1.41% ± 0.60%, which 0.125 mg mL⁻¹ alginate increased CD68 expression to 5.89% ± 0.35%, and addition of pCM reduced it to 2.71% ± 0.63% (*p* = 0.005). Moreover, 1.25 mg mL⁻¹ alginate upregulated CD68 expression on RAW macrophages to 7.64% ± 2.1%, while addition of pCM to the culture reduced it to 2.91% ± 1.20% (*p* = 0.008). It should be noted that RAW cell line does not fully mimic the macrophage responses;^[37] however, in the context of inflammatory response with various stimuli (such as LPS) and cytokine secretion, RAW cells are valuable for assessing macrophage responses.^[38]

To test the validity of these observations under a more potent stimulatory conditions, we used LPS as a strong TLR4 agonist. Figure 3e shows the schematic representation of experimental procedure, where macrophages were incubated with 10 and 100 ng mL⁻¹ of LPS for 12 h in the presence or absence of pCM. LPS activated macrophages with a similar trend as UP-LVG, although the amount of cytokine secretion was higher via LPS activation (Figure 3f), and presence of pCM reduced the TNFα, IL-6, and GM-CSF cytokine secretion. These results suggest that pCM possesses immunomodulatory effects on murine

macrophages when activation is partly through LPS stimulation. The CD68 expression for the culture with 10 and 100 ng mL⁻¹ LPS was 10.96% ± 3.93% and 11.59% ± 2.75%, respectively. Addition of pCM to the culture reduced the CD68 expression to 4.53% ± 1.39% for the former (*p* = 0.0003), and 3.78% ± 1.29% for the later (*p* = 0.009) (Figure 3g,h).

These results in their totality suggest that pCM inhibits alginate- or LPS-induced activation of murine macrophages. We specifically have focused on macrophages due to their critical role in the long-term functionality of the implants. Macrophage depletion by clodrosome (liposomal clodronate) has shown to prevent fibrosis against alginate^[8a,9a] and poly(ethylene glycol)^[39] implants. It is therefore concluded that the anti-fibrotic effect of pCM is partly due to their macrophage suppression.

Given that mouse biology does not always predictably model human biology, and to better understand some of the cellular pathways underlying the reduction of macrophage activation, we further pursued studying the interaction of pCM with human macrophages. Based on our earlier findings here, pCM dampens the secretion of inflammatory cytokines from macrophages that

Figure 4. pCM reduces inflammatory response partly through regulation of NFκB pathway. **a**) Representative IVIS images of the SEAP activity (NFκB activation) as a result of 10 and 100 ng mL⁻¹ LPS stimulation in the presence and absence of pCM. **b**) Luminescence count from IVIS imaging was quantified using equivalent regions on interest, suggesting that pCM is a potent inhibitor of LPS induced NFκB activation. **c**) Same conditions were replicated, and signals were acquired using a plate reader, demonstrating a similar trend in the potency of pCM to inhibit NFκB activation. For instance, addition of pCM to the culture, reduced the luminescence counts of 10 ng mL⁻¹ LPS activated THP-1 cells from 1084.2 ± 186.2 to 116.0 ± 66.5 ($p < 0.0001$). **d**) Study timeline of cathepsin activity measurements in C57/BL6 mice. **e, f**) Representative IVIS images at 7 and 14 days after implantation. **g**) Total radiant efficiency was quantified, where 7 days after implantation, inflammatory response against pCM-Alg mixture ($n = 3$; $2.06 \times 10^8 \pm 1.42 \times 10^8$) was significantly lower than that of CCM-Alg ($n = 6$; $5.61 \times 10^8 \pm 2.06 \times 10^8$), which is the mixture of CCM and alginate. Data are presented as mean $\pm$ SD. Statistical analysis was done by unpaired *t*-test analyses with Welch's correction.

are hallmarks of NFκB pathway (i.e., IL-6, TNFα, GM-CSF, and IL-1β). It is also known that through agonistic interaction with TLR4, LPS activates an IKK complex, phosphorylating IκB proteins. Upon phosphorylation, IκB gets ubiquitinated, which liberates NF-κB/Rel complexes. Activated NF-κB/Rel complexes are further activated by post-translational modifications and translocate into nucleus to induce target gene expression, including IL-6, IL-1, and TNFα in activated macrophages.^[40] We particularly hypothesized that pCM downregulates/interferes with the activation of NFκB pathway. We therefore repeated the stimulation of macrophages in NFκB reporter THP-1 human cell lines, which are integration of an NFκB-inducible secreted embryonic alkaline phosphatase (SEAP) reporter construct. As a result, these cells could quantitatively measure NF-κB activation by determining the activity of SEAP.

We found that pCM reduces the NFκB activation in both 10 and 100 ng mL⁻¹ LPS stimulation. **Figure 4a** shows the representative images of the SEAP activity (NFκB activation) as a result of 10 and 100 ng mL⁻¹ LPS stimulation in the presence and absence of pCM. Luminescence count from IVIS imaging was quantified using equivalent regions on interest (**Figure 4b**), suggesting that pCM is a potent inhibitor of LPS induced NFκB activation. Same conditions were replicated, and signals were acquired using a plate reader, demonstrating a similar trend in the potency of pCM to inhibit NFκB activation (**Figure 4c**). Addition of pCM

to the culture, reduced the luminescence counts of 10 ng mL⁻¹ LPS activated THP-1 cells from 1084.2 ± 186.2 to 116.0 ± 66.5 ($p < 0.0001$).

Different pathways have been associated to biomaterials fibrosis including NFκB,^[41] colony stimulating factor receptor,^[8a,9a] and JAK/STAT.^[9e,42] NFκB regulates multiple aspects of innate and adaptive immunity, and plays a critical role in regulating the function, activation, and survival of innate immunocytes and inflammatory T cells.^[40] NFκB pathway has been reported in response to PDMS,^[9b] poly(ethylene glycol),^[41a] and alginate,^[41b] and reduction in NFκB has been correlated with reduced fibrosis.^[9b,e] Similar to observations in the present study, other works have also reported the reduction in NFκB activation with secretome of MSCs.^[14,43] We therefore could conclude that one mechanism by which pCM-Alg demonstrates lower FBR is due to the reduction of NFκB activation of macrophage on, or around microcapsules.

To further validate the anti-inflammatory effects on pCM-Alg in vivo, we quantified the induction of cathepsin activity around the implants. Cathepsin is a protease that breaks down proteins and peptides in order to eliminate a foreign substrate. Cathepsin activity measurement has been established as a robust method to evaluate the inflammatory response against implanted biomaterials in vivo.^[8a,12,44] Mechanistically, TLR4 stimulation of macrophages has shown to indirectly increase cathepsin

activity.^[45] Cathepsin expression of macrophages increases in response to TLR4 agonist; however, mRNA expression of cathepsin does not change. In addition, neutralizing antibodies against IL-1 β , TNF α , IFN- β could inhibit the cathepsin upregulation. Thus, augmented cathepsin activity is reported to be through involvement of secreted cytokines.^[45]

We thus quantified the cathepsin activity 7 and 14 days after implantation of alginate (Figure 4d–f). To control the effect of culture media on the cathepsin activity measurements, we used our standard CCM addition to the 2.5% alginate solution. We found that 7 days after implantation, inflammatory response against pCM-Alg mixture ($n = 3$; $2.06 \times 10^8 \pm 1.42 \times 10^8$) is significantly lower than that of CCM-Alg ($n = 6$; $5.61 \times 10^8 \pm 2.06 \times 10^8$), which is the mixture of CCM and alginate (Figure 4d,f). Therefore, pCM could reduce the inflammatory response against alginate both in vitro and in vivo.

Our study offers a biohybrid formulation that reduces the inflammatory response against alginate microcapsules. This biohybrid scaffold takes advantage of immunosuppressive capabilities of MSC secretory factors, where loading alginate microcapsules with these factors reduces immune infiltration around microcapsules and decrease the cathepsin activity of alginate in vivo. We found that alginate induces inflammatory response in murine macrophages in a dose dependent manner, leading to secretion of TNF α , IL-6, IL-1 β , and GM-CSF. Addition of pCM to the media reduces the macrophage activation in response to alginate ad LPS, both of which partly activate macrophages through TLR4 pathway.

The pCM-Alg platform benefits from facile preparation method, which could easily be scaled-up for high-throughput manufacturing processed. In addition, through its controlled-release nature, many drug delivery and tissue regeneration applications could be further explored using pCM-Alg. Specifically, pCM-Alg could regulate the fibrotic response against the implants, where other parenchymal cells could be formed around the microcapsules (as shown in Figure 1e,f). This could encourage the tissue regeneration and wound healing around the pCM-Alg platform. Moreover, finding a molecular mechanism underlying the anti-inflammatory effects of secretome (and also extracellular vesicles secreted by stem cells) seem to be very tedious. This complexity mainly originates from the complexity and variety of molecules (including proteins, coding and non-coding RNAs, and lipids) present in the secretome and extracellular vesicles.^[17,19f] To address this complexity, at least partly, we here propose that mechanisms underlying the effect of complex biologics could be characterized through their interactions with activated immunocytes. This interaction could be analyzed with RNAseq of immunocytes, establishing consistency and potency assays for complex biologics.

Experimental Section

Processing Condition Media: Umbilical cord derived MSCs (UCMSCs) were the source of MSCs in this study. UCMSCs were isolated and characterized (Figure S1, Supporting Information), and these cells were the source of conditioned media use throughout the study. To prepare processed condition media (pCM), MSCs were expanded between passages 6–10. After reaching 70% confluency, fresh serum-free media was added to the culture flasks and further cultured for 2 days. Collected media was

then centrifuged at $300 \times g$ and 4°C for 30 min and $16\,500 \times g$ and 4°C for 20 min, which is known to remove dead cells and microvesicles.^[17,19f] Supernatant were then collected and named as processed Conditioned Media (pCM), which was then stored at -80°C until use. It should be noted that maximum storage time was less than 1 year.

EVs Isolation: As previously described,^[17] conditioned media from cultures of MSC cells grown as described above were centrifuged at $300 \times g$ for 10 min. Supernatant was collected and transferred to ultracentrifuge tubes (Polyallomer Quick-Seal centrifuge tubes 25×89 mm, Beckman Coulter). Samples were then centrifuged in a Beckman Coulter ultracentrifuge (Optima L-90 K or Optima XE-90 Ultracentrifuge, Beckman Coulter) for 20 min at $16\,500 \times g$ (Type Ti 45, Beckman Coulter), to remove microvesicles. Supernatant was then carefully collected and centrifuged for 2.5 h with a Type 45 Ti rotor at 4°C at $120\,000 \times g$. EVs pellet was resuspended in PBS and stored at -80°C .

Alginate Microcapsules Preparation: UPLVG alginate (NovaMatrix, Sandvika, Norway) were fabricated by dissolving 2.5% w/v in 0.9% sterile saline solution and mounted on an air-driven electrostatic microcapsule generator (Nisco Engineering Inc., Oslo, Norway). The alginate solution was added dropwise into a sterile filtered ($0.22\ \mu\text{m}$) gelling solution composed of sterile 20 mM barium chloride and 25 mM HEPES solution to generate circular microcapsules of ≈ 350 microns in diameter. Alginate microcapsules loaded with pCM (pCM-Alg) microgels were prepared by lyophilization technique overnight. Microcapsules were first centrifuged at $100 \times g$ and 4°C for 5 min. Supernatant was discarded and $500\ \mu\text{L}$ of a 10% PEG 400 and 8% glycerol solution per 3000 microcapsules was added. Samples were then frozen at -80°C for 4 h and lyophilized for 12 h. Lyophilized microcapsules were then rehydrated by pCM (or cell culture media) on a rocking plate at 4°C for 4 h with constant agitation at 500 rpm. Next, pCM-Alg microcapsules washed with sterile saline solution in triplicate for 1 min each. Ctrl-Alg and CCM-Alg microcapsules were also further subjected to these steps and rehydration step was conducted with sterile saline solution and sera-free culture media, respectively.

Microscopy: Sections were then cut into $5\ \mu\text{m}$ slices and blocked with 2% rat serum and 2% BSA. Slides were then stained with DAPI (1:1000), AlexaFluor 647 anti- α SMA (1:200), and FITC anti-CD68 antibodies (1:200). Total area of DAPI, CD68, and α -SMA were quantified using ImageJ (NIH, USA) and compared between AI and control group using unpaired *t*-test with Welch's correction (n.s., non-significant; $*p < 0.05$). To quantitatively compare the number of infiltrated cells, signal area was measured in similar magnifications and normalized by the total tissue area in the image.

Controlled Release Studies: Both pCM-Alg and CCM-Alg microcapsules (≈ 1000) were incubated at 37°C in a humidified incubator with 5% CO_2 for 48 h. At indicated timepoints after co-incubation (i.e., 8, 24, and 48 h), 1 mL of the culture supernatant were collected and 1 mL of sterile DPBS was replaces. Isolated proteins were then measured for protein quantity, and EVs content. The results from protein quantity analyses were then depicted as total protein release versus time. Collected media from the cultures were then subjected to exosome isolation kit (ExoQuick ULTRA EV Isolation Kit CAT#: EQUltra-20A-1) and isolated EVs were then frozen at -80°C until analysis. To count and visualize released EVs, Nanoparticle Tracking Analysis (NTA) was performed using the Nanosight system (NS300 Malvern instruments, USA). EVs were analyzed based on light scattering using an optical microscope aligned perpendicularly to the beam axis. Continuous syringe pump flow was adjusted to be $100\ \mu\text{L}\ \text{min}^{-1}$. A 60 s video was recorded and subsequently analyzed using NTA software.

In Vitro Cultures and Cytokine Analyses: RAW 264.7 cells were purchased from ATCC (CAT# TIB-71) and NF κ B reporter Luc-THP-1 human cell lines were purchased from InvivoGen (CAT#: thpl-nfkb) employed for downstream experiments of this study. Passages 5–10 were cultured in RPMI 1640 supplemented with 10% of heat inactivated FBS in the presence of 1% penicillin/streptomycin and 1% L-glutamine. Cells were then stimulated with 10 and $100\ \text{ng}\ \text{mL}^{-1}$ of LPS (Invitrogen, CAT#: 50-112-2025). In addition to LPS, 0.125 and $1.25\ \text{mg}\ \text{mL}^{-1}$ of alginate were used for stimulation. Stimulated and non-stimulated cells were then mixed with diluted pCM (50%). To eliminate the possibility of media and/or growth factor effect on the authors' analyses, pCM was mixed with fresh culture

media (1:1 ratio) and control media was prepared using culture media and sterile DPBS (1:1 ratio). Control cells, LPS stimulated cells in the presence and absence of 50% pCM, and non-stimulated cells in the presence and absence of 50% pCM (100 000 cells for each condition) were cultured for 10–14 h at 37 °C in a humidified incubator with 5% CO₂. Next, both cells and supernatant were collected for flow cytometry and cytokine analyses, respectively. Supernatants were centrifuged at 2500 × g and 4 °C for 5 min and stored at –80 °C. Samples were then shipped on dry ice to Eve Technologies (Calgary, Canada), where cytokines were analyzed using Mouse Focused 10-Plex Discovery Assay (MDF10).

Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats from healthy, anonymous blood donors (UCI institute for clinical and transitional science) by density gradient centrifugation (Ficoll-Paque plus, GE Healthcare). Dynabeads Human T-Activator CD3/CD28 for T Cell Expansion and Activation was used with 1:1 ratio of PBMCs:Dynabeads. The pCM was mixed with fresh culture media (1:1 ratio) and control media was prepared using culture media and sterile DPBS (1:1 ratio). Dynabeads were then added to isolated PBMCs in the presence and absence of pCM. Supernatants were collected and Luminex assay was used to analyze the secreted cytokines. 50 µL of PBMC culture supernatants were collected and either frozen at –80 °C or immediately analyzed using a human custom ProcartaPlex (11plex, ThermoFisher Scientific, Vienna, Austria) with Luminex 77. Results were then reported as Mean Fluorescence Intensity (MFI).

Flow Cytometry: As described earlier, RAW 264.7 cells were stimulated with either LPS (10 and 100 ng mL^{–1}) or alginate (0.125 and 1.25 mg mL^{–1}) in the presence and absence of 50% pCM. 100 000 cells for each condition were cultured for 10–14 h at 37 °C in a humidified incubator with 5% CO₂. Next, cells were collected and stained with 7AAD (1:1000) and CD68 (1:200).

Western Blotting: 1 mL of released proteins from pCM-Alg and CCM-Alg were collected and subjected to exosome isolation using (ExoQuick ULTRA EV Isolation Kit CAT#: EQUltra-20A-1). Around 20 µg of the released proteins were mixed with 1X RIPA (Cell signaling technologies, USA) buffer and sonicated for 5 min, three times, with vortexing in between. Protein contents were measured using a BCA protein assay kit (Thermo Scientific Pierce, Rockford, IL, USA). 25 µL of BSA standard or 25 µL of sample were transferred to a 96-well plate, and 200 µL working reagent was added. The plate was incubated for 30 min at 37 °C and absorbance was analyzed with a SpectraMax 384 Plus spectrophotometer at 562 nm and the SoftMax Pro software (Molecular Devices, 1311 Orleans Drive, Sunnyvale, CA, USA). 20 µg of protein from each sample was then separated on a gradient precast polyacrylamide gel (Mini-PROTEAN; Bio-Rad laboratories, Hercules, CA, USA). Samples were then transferred onto a nitrocellulose membrane which was then blocked with 5% Blotting Grade Blocker Non-Fat Dry Milk (Bio-Rad Laboratories) in Tris-buffer saline supplemented with %0.1 Polysorbate 20 (TBST) overnight at 4 °C. Membrane was then washed with TBST incubated with primary antibodies against Calnexin (1:1000; clone H-70; Santa Cruz Biotechnology, Santa Cruz, CA, USA), Galectin-1/LGALS1 (D608T) Rabbit mAb (Cat# 12936), and TSG101 (1:1000; clone 4A10; Abcam, Cambridge, UK) dissolved in 0.25% Blotting Grade Blocker Non-Fat Dry Milk in TBST overnight at 4 °C. Next, membrane was washed with TBST for 10 min, three times. Secondary antibodies (Calnexin: (1:2500) ECL anti-rabbit IgG horseradish peroxidase-linked F(ab')₂ fragment (donkey, anti-rabbit); for TSG101: (1:2000) ECL anti-mouse IgG horseradish peroxidase-linked F(ab')₂ fragment (sheep, anti-mouse); GE Healthcare, Buckinghamshire, UK) were diluted in 0.25% Blotting Grade Blocker Non-Fat Dry Milk in TBST and incubated for 1.5 h. Membranes were analyzed with ECL Prime Western Blotting Detection (GE Healthcare) and a VersaDoc 4000 MP (Bio-Rad Laboratories).

IVIS Imaging: NFκB reporter Luc-THP-1 human cell lines were (InvivoGen, San Diego USA) used which are integration of an NFκB-inducible secreted embryonic alkaline phosphatase (SEAP) reporter construct. These cells were engineered THP-1 monocyte cell line by stable integration of an NFκB-inducible Luc reporter construct. The levels of NFκB-induced secreted luciferase in the cell culture supernatant were readily assessed with Quanti-Luc (CAT#: rep-qlc2). As a result, these

cells could quantitatively measure NFκB activation by determining the activity of SEAP. Cells were cultured in a phenol-free media and supernatants (as described in the in vitro culture section of the methods) were collected. QUANT-Luc assay solution was added with a concentration of 1 mg mL^{–1} and incubated for 30 s. The resulted plate was then imaged in an IVIS imager (or VersaDoc 4000 MP). Exposure time was adjusted as 0.2 s, field of view 12.5, f number 16, and binning factor of 4 were selected as optimized acquisition settings.

To evaluate the inflammatory response against alginate, live animal imaging was conducted using IVIS imager to measure the cathepsin activity.^[44] Mouse were fed alfalfa-free meal to eliminate the fluorescence background. To minimize the inflammatory response due to surgical procedure, and to characterize the inherent inflammatory response against alginate, 180 µL of %2.5 alginate was mixed with 20 µL of PBS, CCM, or pCM. Each sample was then subcutaneously injected into opposite diagonal spaces on the mid-back of the male C57/BL6 mice (Figure 4d). 6 days after injection, 100 µL of ProSense 750 FAST (NEV11171, PerkinElmer Inc.) was injected intraperitoneally 18–24 h prior to image capturing session. Whole-body IVIS imaging was then performed, and signals were quantified using equivalent regions of interest. At days 7 and 14, mice were scanned by IVIS Spectrum system (Xenogen, Caliper LifeScience). The mice were anesthetized with 2% isoflurane and oxygen and maintained at 1.5% isoflurane throughout the procedure. The settings of the IVIS Spectrum system were exposure = 9.0, binning = medium, F-Stop = 4, excitation = 710 nm, and emission = 780 nm.

Animal Studies: All animal procedures were performed under approved University of California Irvine, Institutional Animal Care and Use Committee (Protocol #: AUP-17-241). Around 3000 of pCM-Alg or CTRL-Alg microcapsules were implanted subcutaneously into 12–16 weeks old C57/BL6 male mice for 2 weeks. The mice were anesthetized using 2% Isoflurane USP-PPC (Pharmaceutical partners of Canada, Richmond, ON, Canada). The mouse back hairs were shaved mouse skin sterilized using ENDURE 400 Scrub-Stat4 Surgical Scrub (chlorhexidine gluconate, 4% solution; Ecolab Inc., Minnesota, USA) and 70% ethanol. Surgical procedures were conducted under a sterile laminar flow hood and sterility measures were strictly upheld to minimize possible surgery-induced infection or severe inflammation. To implant the scaffolds, one 3–5 mm incision was cut on the dorsal upper section of each mouse. Around 3000 microcapsules were dispersed in 500 µL sterile PBS and injected into the subcutaneous pocket within bottom dorsal section of each mice. The incisions were then sutured using Surgipro II monofilament polypropylene 6–0 (Covidien, Massachusetts, USA) and sterilized with Betadine (a 10% povidone-iodine solution for skin disinfection) to prevent possible infections. Additionally, buprenorphine (as HCL) (0.03 mg mL^{–1}; Chiron Compounding Pharmacy Inc. Guelph, ON, Canada) was added to water bottles in the cages as a pain reliever. All animals were then carefully monitored for 5 days as the standard post-operational procedure. Upon explantation, capsules were fixed in 4% formaldehyde at 4 °C overnight and embedded in paraffin. Whole mount explants were also stained and embedded on poly-L-lysine coated glass slides for confocal microscopy.

Statistical Analysis: Data were plotted as mean ± standard deviation (SD) and analyzed with the software GraphPad Prism. Statistical analysis was conducted using unpaired t-test with Welch's correction ($n = 3$ or 4 as indicated per experiment). Differences were considered statistically significant if the p -value < 0.05).

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

alginate, exosomes, foreign body response, inflammation, macrophages, mesenchymal stem (stromal) cells, secretome

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