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Alpha-2-macroglobulin loaded microcapsules enhance human leukocytes functions and innate immune response

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ABSTRACT

Synthetic microstructures can be engineered to deliver bioactive compounds impacting on their pharmacokinetics and pharmacodynamics. Herein, we applied dextran-based layer-by-layer (LbL) microcapsules to deliver alpha-2-macroglobulin (α 2MG), a protein with modulatory properties in inflammation. Extending recent observations made with dextran-microcapsules loaded with α 2MG in experimental sepsis, we focused on the physical and chemical characteristics of these microstructures and determined their biology on rodent and human cells. We report an efficient encapsulation of α 2MG into microcapsules, which enhanced i) human leukocyte recruitment to inflamed endothelium and ii) human macrophage phagocytosis: in both settings microcapsules were more effective than soluble α 2MG or empty microcapsules (devoid of active protein). Translation of these findings revealed that intravenous administration of α 2MG-microcapsules (but not empty microcapsules) promoted neutrophil migration into peritoneal exudates and augmented macrophage phagocytic functions, the latter response being associated with alteration of bioactive lipid mediators as assessed by mass spectrometry. The present study indicates that microencapsulation can be an effective strategy to harness the complex biology of α 2MG with enhancing outcomes on fundamental processes of the innate immune response paving the way to potential future development in the control of sepsis.

Key words:

Alpha-2-macroglobulin; LbL Microcapsules; Inflammation; Leukocyte Activation; Phagocytosis.

INTRODUCTION

A promising method to provide controlled, sustained delivery and release of proteins is the layer-by-layer (LbL) microencapsulation technique [1]. Several studies have focused on the construction of nano- and micro- capsules engineered as carriers for active compounds including, enzymes, nucleic acids, proteins and chemo-therapeutics. These protocols enact a drug-delivery system to carry controlled quantities of a therapeutic payload to a specific target site or tissue. Their main advantages are versatility, control over function and response-tailored to capsule structure. Multi-compartmental structure may allow inclusion of various compounds at defined doses in a single vesicle, altering their activity and accessibility to environment [2-4]. Extensive research of microcapsules (MCs) delivery shows internalization by target cells without overt toxicity. Microcapsules made of biodegradable polymers can degrade over time to gradually release encapsulated compounds, a phenomenon reported both *in vivo* and *in vitro* [5]. All these characteristics make microcapsules a versatile delivery tool, amenable to the delivery of proteins that can modulate the inflammatory process.

Most proteins have short half-life when applied *in vivo*, requiring multiple administrations. Encapsulation often yields i) amelioration of bioactions, ii) enhancement of therapeutic efficacy by delivery to a specific tissue and iii) delivery across biological barriers. Among the

mediators of the inflammatory process, the acute phase protein alpha-2-macroglobulin (α 2MG) is of particular interest. α 2MG acts as a protease inhibitor and carrier for several growth factors and cytokines, including TNF- α , IL-1 β , IL-6 and TGF- β [6]. Activation of α 2MG results in the entrapment of proteases with the entire complex now being able to bind to the low-density lipoprotein receptor like protein-1 (LRP-1; [7]), the α 2MG receptor. Therefore, the α 2MG-LRP-1 pair has a great potential for the regulation of cytokine homeostasis in blood and tissue, a critical point in the pathogenesis of several diseases.

We have recently showed that α 2MG is abundant in a specific subset of neutrophil-derived vesicles (called microparticles) [8], and to be a major determinant for their protective effects in experimental sepsis [9]. Soluble α 2MG has a short systemic half-life in mice (~4 min) [10] being mainly cleared by the liver [11]. To maximize α 2MG protective activity and study these effects in the absence of other proteins present in the natural vesicles, we established if synthetic microcapsules could recapitulate the biological functions of α 2MG. Biodegradable microcapsules were generated with a layer-by-layer microencapsulation technique and loaded with α 2MG. In a model of peritoneal sepsis the synthetic α 2MG-microcapsules controlled bacterial load, leading to animal survival [9]. These initial experiments provided important proof-of-concept that manufacturing microcapsules enriched with α 2MG was a viable strategy to replicate the bioactions of α 2MG when present in natural microvesicles. However, little is known about the interaction and properties of the synthetic microcapsules with human primary cells. Herein, we focused on the physical and chemical characteristics of these new biodegradable microcapsules loaded with α 2MG and have investigated their interaction and biological functions in human cells and experimental settings, revealing, for the first time, their translational potential for therapeutic approaches.

MATERIALS AND METHODS

Please refer to the Supplementary Material for details on protocols, materials and sources.

α 2MG enriched-microcapsule generation

Microcapsules (MCs) were prepared according to LbL assembly technique by alternate deposition of oppositely charged polyelectrolytes on sacrificial calcium carbonate template microparticles (see Figure 1 for schematic) [4]. α 2MG was incorporated into the cores by co-precipitation at particles synthesis stage, as described [9]. As a control, an empty preparation of MCs was used. Positively charged PLA and negatively charged DS were used for shell assembly and adsorbed from 2 mg/ml solutions in 0.15M NaCl. One middle layer of FITC-PLL was adsorbed instead of PLA, used for the rest of positively charged layers, to fluorescently label microcapsules for confocal visualization and flow cytometry measurements. The final shell structure obtained was PLA/DS/FITC-PLL/[DS/PLA]₂ with positively charged outermost layer of PLA. After the shells were fully constructed, CaCO₃ cores were dissolved in 0.2 M EDTA (pH 6.5). To estimate the encapsulation efficiency, supernatants were collected from particles synthesis, from the first three layers depositions and particles dissolution steps (named A0, A1, A2, A3, AE).

α 2MG enriched-microcapsules characterization

Microcapsules morphology was characterized using FEI Inspect F scanning electron and Leica TS confocal microscopes. MCs were counted (obtaining values of 425×10^6 and 264×10^6 capsules/ml for $\alpha 2$ MG- and empty-MCs, respectively) and analyzed by Flow Cytometry with BD LSRFortessa, together with $1 \mu\text{m}$ beads for comparison. The content of $\alpha 2$ MG was assessed by Western blot analysis in $\alpha 2$ MG-MCs, empty-MCs and supernatants from preparation steps (A0, A1, A2, A3, AE), loading soluble $\alpha 2$ MG for comparison. To assess the efficiency of encapsulation, un-loaded protein was quantified by inverted ELISA. Standards (0.005 – $5 \mu\text{g/ml}$ of active $\alpha 2$ MG) and supernatants A0, A1, A2, A3 and AE were i) incubated overnight at 4°C ; ii) an anti- $\alpha 2$ MG ($1:50.000$; clone $\square 2\text{-M}\square 1$ IIE7; BioMac) was applied for 2h RT; iii) after washing and incubation with anti- mouse HRP-conjugated antibody ($1:5000$; Invitrogen) for 2h, $3,3',5,5'$ -Tetramethylbenzidine (TMB) substrate buffer (R&D System) was added for 30 min; iv) the reaction was stopped with 1N sulphuric acid (Sigma) and v) absorbance read at 450 nm with a fluorescence plate reader.

In vitro biological analyses

Preparation of human peripheral monocytes, monocyte-derived macrophages (MDM) and neutrophils. Peripheral blood neutrophils and monocytes were freshly isolated as described [12]. Purified monocyte population was obtained by adhesion (1h , 37°C , 5% CO_2) and monocyte-derived macrophages (MDM) were prepared from monocytes, by culture ($8\text{--}10$ days) in RPMI 1640 containing 20% fetal bovine serum (FBS), glutamine and antibiotics [12].

Flow chamber assay. To assess leucocyte-endothelial interaction, primary human umbilical vein endothelial cells (HUVEC) were collected and plated overnight in μ -Slides VI^{0.4} (Ibidi™) [13, 14]. The confluent monolayers were stimulated with $\text{TNF-}\alpha$ (10 ng/ml) in complete medium (M199) 0% FBS (to avoid contamination of exogenous $\alpha 2$ MG), in presence or absence of different amounts of MCs. Neutrophils were incubated for 10 min at 37°C , and then perfused over endothelial cells at 1 dyne/cm^2 for 8 minutes [14]. In another set of flow experiments, exogenous active $\alpha 2$ MG was applied (9.4 ng/slide).

Confocal microscopy. To visualize MCs and endothelial cell interaction, HUVEC and flown neutrophils were stained with Alexa Fluor® 546-Phalloidin (5 U/mL , Invitrogen) and left in Probing Antifade medium (Invitrogen) containing DAPI. They were visualized using a Zeiss LSM 510 META scanning confocal microscope and analyzed by Zeiss LSM Imaging software (Carl Zeiss). In another set of experiments, cells were stained with Alexa Fluor® 633-Wheat Germ Agglutinin ($1 \mu\text{g/ml}$; Invitrogen) followed by anti-active $\alpha 2$ MG antibody ($10 \mu\text{g/ml}$, clone $\square 2\text{-M}\square 1$ IIE7, BioMac), Alexa Fluor® 594 secondary antibody (Invitrogen) and Probing Antifade medium (Invitrogen) containing DAPI. By acquiring Z-stack images, the number of $\alpha 2$ MG-positive particles on the membrane surface were acquired and counted in each sample using NIH ImageJ 1.48 software.

Flow cytometry. Monocytes and MDM were assessed for both surface and intracellular expression of $\alpha 2$ MG receptor (LRP1 or CD91, $5 \mu\text{g/ml}$, clone A2Mr alpha-2, AbDSerotec) along with the lineage specific lineage marker: CD14 ($0.5 \mu\text{g/ml}$, clone 61D3, eBioscience) for monocytes and CD68 ($0.5 \mu\text{g/ml}$, clone Y1/82A, eBioscience) for MDM. Cells were then analyzed with a FACSCalibur flow cytometer using CellQuest TM and FlowJo software.

Phagocytosis assay. MDM were evaluated for their ability to phagocytose Zymosan and *Escherichia Coli* (*E. Coli*) particles. MDM were incubated with different amounts of α 2MG- or empty-MCs for 24 h (at 37°C, 5% CO₂). Zymosan (Zymosan A from *Saccharomyces Cerevisiae*) and *E. Coli* particles (Strain K12) were conjugated with a fluorescent dye (Bodipy® 576/589, 1 μ M; Invitrogen). After 24 h of incubation with MCs, 125 μ g/ml of fluorescent Zymosan particles or 1 mg/ml of fluorescent *E. Coli* particles were added to the medium for a further 20 min or 1 h, respectively (at 37°C, 5% CO₂). The number of fluorescent phagocytized particles was determined with a fluorescence plate reader (BMG Labtech) and analysed using MARS Data Analysis Software. Cells were further analysed by scanning confocal microscope. To further corroborate our phagocytosis results and discriminate between ingested and membrane-bound particles, human macrophages were incubated with microcapsules (1x10⁵/well) or soluble α 2MG (94ng/well) as described above and then incubated with phRodo *E. Coli* (1mg/ml, Invitrogen) for 30 min (37°C, 5% CO₂), following manufacture's instructions. The fluorescent emission of internalized particles was analyzed by Flow cytometry (FACSCalibur using CellQuest TM and FlowJo software). In another set of experiments Bodipy®-*E.Coli* particle phagocytosis was monitored in biogel-elicited mouse macrophages following the same protocol above.

In Vivo studies

C57Bl/6 mice (male, 6-8 weeks; Charles River) were used.

Acute Peritonitis. Vehicle (PBS), empty MCs (1x10⁵/mouse), α 2MG-MCs (1x10⁵/mouse) or equivalent levels of soluble active α 2MG (94 ng/mouse) were administered i.v. followed by i.p. administration of Zymosan A (0.1 mg). Peritoneal lavages were collected after 4h and leukocyte infiltration was assessed by light microscopy, followed by differential analysis using anti-Gr-1 and anti-F4/80 staining and flow cytometry analysis.

In vivo phagocytosis. Mice were injected with 1 ml of 2% Bio-Gel (Bio-Rad) i.p. and 3 days later, vehicle (PBS), empty MCs (1x10⁶/mouse), α 2MG-MCs (1x10⁶/mouse) or soluble α 2MG (940 ng/mouse) were administered i.p. After 18 h, mice were injected with fluorescent- (Bodipy® 576/589, 1 μ M; Invitrogen) Zymosan A (1.6 mg i.p.) and peritoneal lavages were collected after 30 min. The fluorescence of engulfed particles in macrophages was evaluated by flow cytometry.

Bioactive lipid quantification. Quantification of Protectin DX (PDX), Leukotriene B₄ (LTB₄), Prostaglandin E₂ (PGE₂), 5-Hydroxy Eicosatetraenoic acid (5-HETE), 15-Hydroxy Eicosatetraenoic acid (15-HETE), 14-Hydroxy Docosahexaenoic Acid (14-HDoHE), 17-Hydroxy Docosahexaenoic Acid (17-HDoHE), 18-Hydroxy Eicosapentaenoic acid (18-HEPE) in peritoneal lavages, after phagocytosis assay, was achieved by LC-MS/MS measurements as described [15]. For each standard, calibration curves were built using 10 solutions at concentration ranging from 0.95 ng/ml to 500 ng/ml.

Statistical analysis

All statistical analyses were performed using GraphPad Prism (v6.0, San Diego CA, USA). Data are expressed as mean \pm SEM of “n” independent experiments. Statistical evaluation

RESULTS

In general, 1×10^6 $\alpha 2\text{MG}$ -MCs contained approximately 1 μg of $\alpha 2\text{MG}$. Furthermore, the majority of $\alpha 2\text{MG}$ was lost in the initial step of incorporation as evident from detection in the A0 supernatant (Figure 1D; right blot). Quantitative data were obtained by ELISA: congruently with the Western blotting data, only the A0 supernatant samples contained $\alpha 2\text{MG}$. We could calculate approximately 6.4 μg of unloaded protein, which is a minimal portion of the total amount of protein used for encapsulation (800 μg), yielding a calculated encapsulation of 94ng of $\alpha 2\text{MG}$ for 100,000 capsules. These microcapsules were tested in two systems where natural vesicles enriched with $\alpha 2\text{MG}$ displayed bioactivity [9].

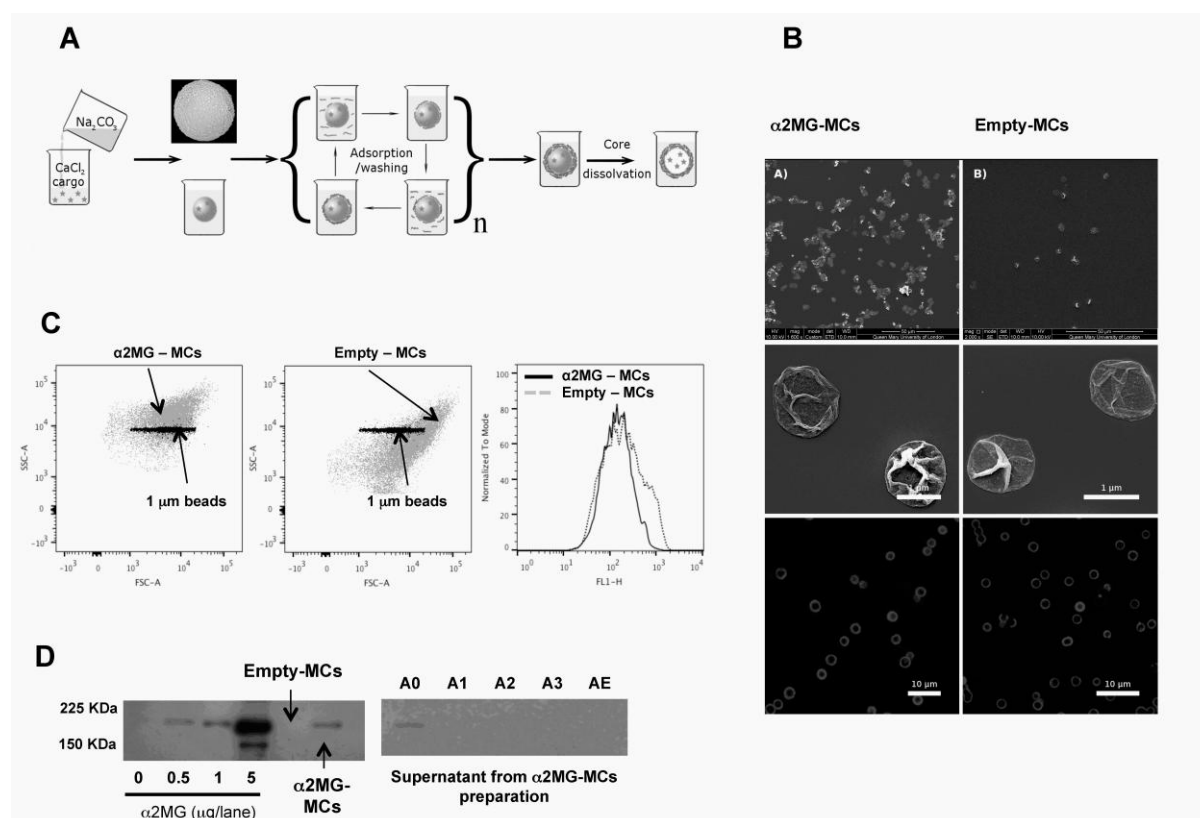


Figure 1. MCs preparation and characterization. (A) MCs were generated using the layer-by-layer assembly protocol by alternate deposition of oppositely charged polyelectrolytes on sacrificial calcium carbonate template particles. (B) Morphology of α2MG-MCs (right panel) and empty-MCs (left panel) as shown by scanning electron and confocal microscopes. (C) Flow-cytometer analysis; forward and side scatter plots (left and middle panels): MCs (grey cloud); 1 μm beads (black cloud). Histograms (right panel): green fluorescence associated with the microcapsules. (D) Western Blot analysis of α2MG content in MCs in comparison with soluble protein.

Biological effects of microcapsules

First we tested α2MG-microcapsules and empty-microcapsules in the flow chamber assay with human neutrophils and human umbilical vein endothelial cells to corroborate the hypothesis that α2MG entrapped in synthetic structure retained its ability to promote cell-to-cell interaction. Thus, different amounts of α2MG-microcapsules, or empty-microcapsules, were incubated with TNF-α-stimulated endothelial cells for 4 h. Following flow at 1 dyne/cm² of freshly isolated human peripheral blood neutrophils, a good extent of white blood cell adhesion was quantified with a significant effect of the capsules at 0.1x10⁵ dose (51±6, 36±5 and 28±3 adherent cells with 0.1x10⁵ α2MG-microcapsules, empty-microcapsules or vehicle, respectively; **p<0.01). Figure 2A illustrates the concentration-response experiments, whereas Figure 2B presents cumulative data for α2MG-microcapsules, empty-microcapsules and soluble α2MG (added to an equivalent amount of 9.4 ng). Confocal analyses of slides at the end of the 8 min flow experiment displayed microcapsule interaction with the human cells (Figure 2C). Image reconstruction in Figure 2D shows the chiefly insertion of the microcapsules within endothelial cells (white arrows). Collectively these

results demonstrate that encapsulation technology ameliorates the pharmacological profile of α 2MG at least within these experimental settings.

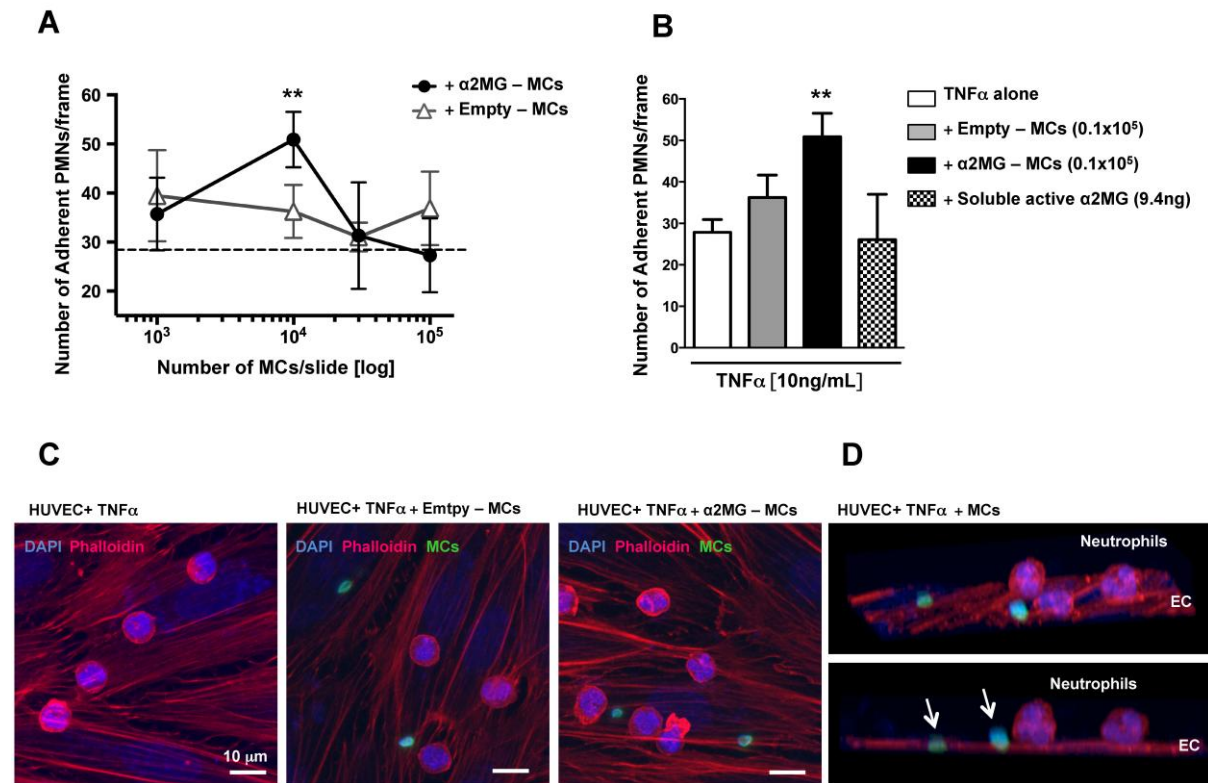


Figure 2. MCs interaction with human endothelial monolayer. (A) Endothelial cells were treated with TNF- α (10ng/mL; 4 h) in presence or absence of different amount of MCs (1×10^3 - 1×10^5) before neutrophils flow (see Methods for protocol details). (B) MCs were compared to an equivalent amount of soluble α 2MG (9.4ng). Data are mean \pm SEM of 9 different human donors for MCs experiments, 3 for soluble α 2MG (One way ANOVA, Bonferroni post-test, ** $p < 0.01$ vs. vehicle). (C) Confocal images of endothelial monolayer treated with TNF- α and then vehicle (left panel), empty-MCs (central panel) and α 2MG-MCs (right panel). Images show cells stained with Alexa Fluor® 546-Phalloidin (red) and DAPI (blue). Microcapsules are in green. (D) 3D images of α 2MG-MCs slide to demonstrate capsule insertion onto the endothelial monolayer (arrows). Images are representative of three distinct analyses.

Next we wanted to visualize deposition of the protein on the human cells. To this aim, neutrophils and endothelial monolayers were stained with agglutinin along with a specific anti- α 2MG antibody against the active conformation of the protein. Figure 3A reports these images. Wheat germ-Agglutinin recognizes sialic acid and N-acetylglucosaminyl residues on the plasma membrane, hence allowed us to analyze specifically the surface of endothelial cells; herein, a significant deposition of α 2MG could be visualized (Figure 3A). Two further notes are worthwhile. First, basal immunostaining for α 2MG was evident in control settings and following incubation with empty microcapsules. Second, this was not particularly different between the two conditions whereas significant accumulation was quantified after α 2MG-MCs (Figure 3B). The incremented α 2MG-immunostaining is not solely associated with the fluorescent capsules, indicating a potential release of the protein on the endothelial cell surface, possibly providing opportunity to interact with the recruited neutrophils.

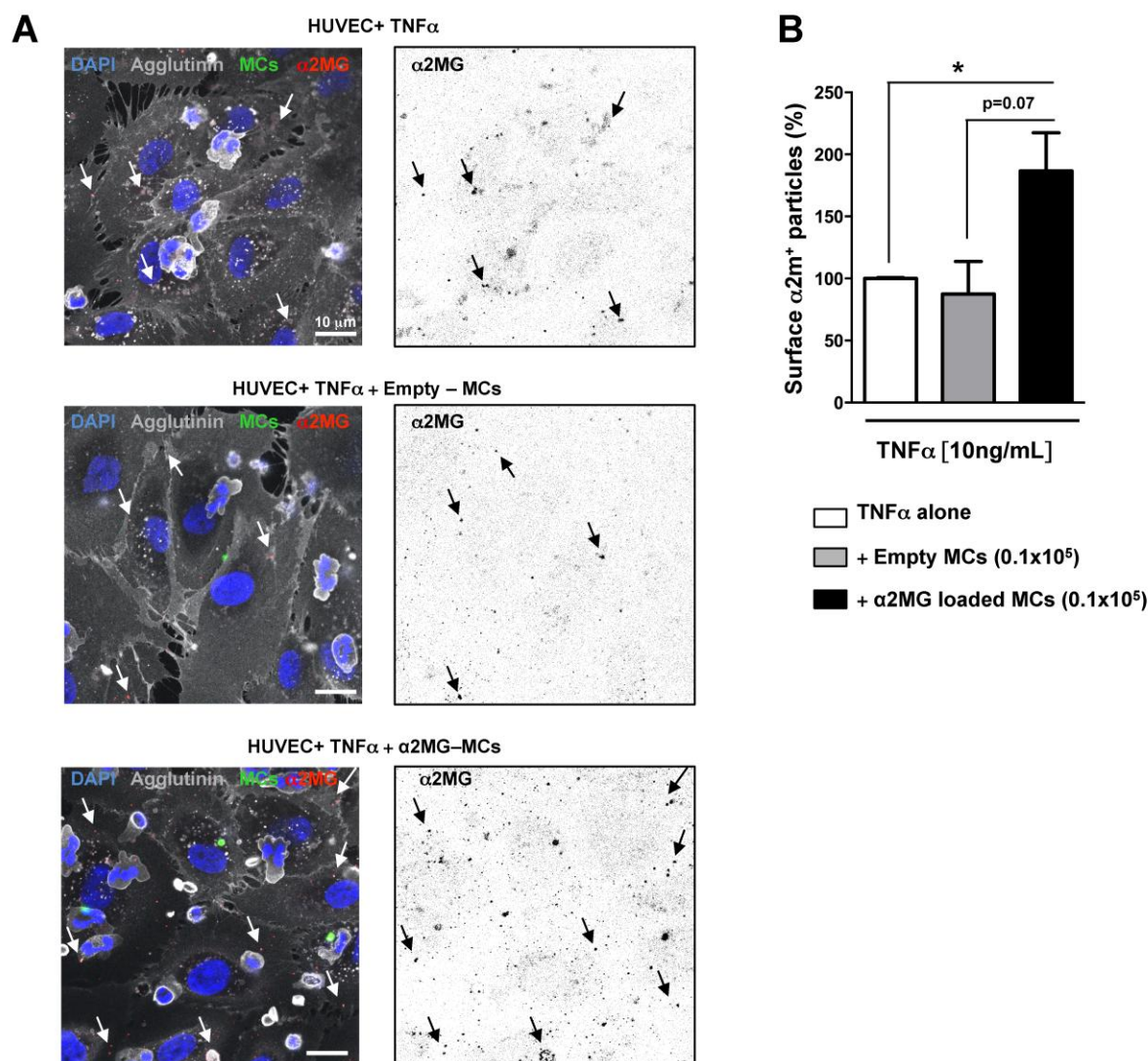


Figure 3. α 2MG-microcapsules promote deposition of active α 2MG on the surface on human endothelial cells. (A) Confocal images of endothelial monolayers treated with TNF- α as in Figure 2, and then vehicle (upper panel), empty-MCs (central panel) or α 2MG-MCs (bottom panel). Cells were stained with Alexa Fluor® 633- Wheat germ-Agglutinin (grey), followed by anti-active α 2MG antibody (red) and DAPI (blue). The intact microcapsules are in green. Z-stack images were acquired and the number of α 2MG-positive particles on the endothelial membrane surface was counted in each sample (right panel for each group, black spots). Images are representative of three distinct analyses. (B) Cumulative data for α 2MG-positive particles as determined by imaging software. Data are mean \pm SEM of 3 independent experiments (Student's *t* test, **p*<0.05 vs. vehicle).

Next we determined if α 2MG-MCs affected human macrophage phagocytosis. The putative α 2MG receptor LRP1 is detected on human neutrophils [9], thus we determined its expression on human monocytes and monocyte-derived macrophages (MDM). LRP-1 (CD91) is generally recognized as a receptor critical for efferocytosis [16]. FACS analysis with an anti-CD91 antibody, along with the lineage specific markers CD14 for monocytes and CD68 for macrophages, revealed a predominant intracellular expression with augmented overall expression in the latter cell type (Figure 4A). Thus, MDM were incubated for 24 h with α 2MG-MCs or empty-MCs, prior to addition of two distinct phagocytic stimuli. As

depicted in Figure 4B, incubation of MDM with α 2M-MCs significantly promote their ability to phagocytose zymosan particles: this effect was concentration-dependent at 1×10^5 and 0.3×10^5 α 2MG-MCs. A similar outcome was obtained with dead *E. Coli* particles, though higher increments over soluble α 2MG could be calculated (e.g. $220 \pm 20\%$, $134 \pm 16\%$ and $139 \pm 11\%$ increased phagocytosis over vehicle for 1×10^5 α 2M-MCs, 1×10^5 empty-MCs and 94 ng soluble α 2MG, respectively; Figure 4C). These effects were not due to alteration in cell viability as confirmed with human MDM (not shown) and RAW 246.7 cells (Supplementary Figure 1A). Moreover, using pH sensitive *E.coli* particles we corroborated these results in human macrophages, demonstrating again a significantly increase of particle uptake in α 2M-MCs-treated cells as compared to empty capsules and soluble protein treatments ($>50\%$, $*p < 0.05$, Supplementary Figure 2). Capsules entered into human MDM cell cytoplasm, as seen by confocal analysis, an effect independent from α 2MG (Figure 4D). Intact MCs could be detected even after the 24 h of incubation (Figure 4D). Interestingly, light microscopy imaging indicated that the distribution of capsules was not homogenous among macrophages with evidence for a subset of cells that internalized one or more capsules, and others devoid of fluorescent material (Figure 4E). This phenomenon is not novel (as seen in RAW 246.7 cells with polystyrene particles [17]) and it is more noticeable in low magnification (Supplementary Figure 3).

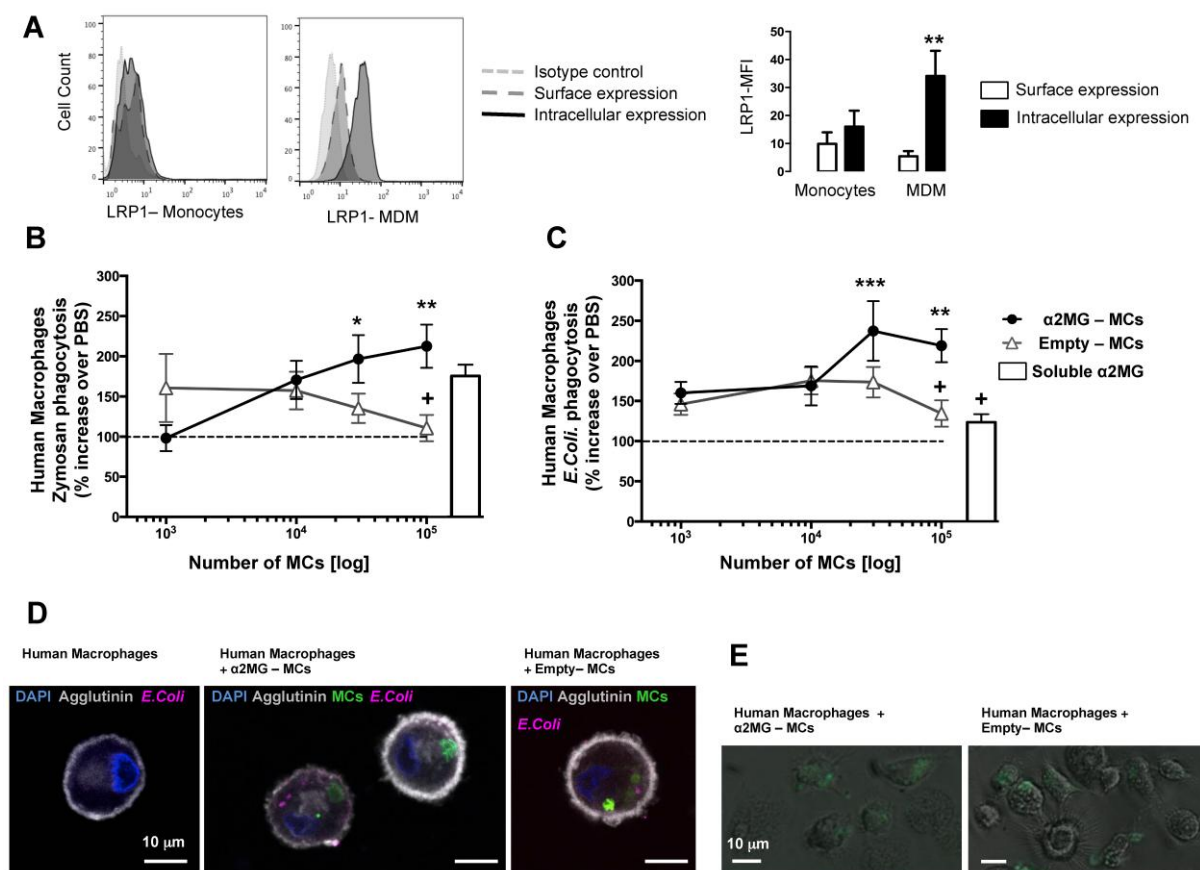


Figure 4. α 2MG-MCs promote monocyte-derived macrophages (MDM) uptake of Zymosan and *E.Coli* particles. Expression of LRP1 on human monocytes and MDM as quantified by flow cytometry in intact and permeabilised cells, along with CD14 for monocytes (0.5 μ g/ml) and CD68 (0.5 μ g/ml) for MDM. (A)

Representative histograms showing augmented levels LRP1 expression on MDM; bar graphs show cumulative data (mean \pm SEM, n= 4; One way ANOVA, Bonferroni post-test, $**p<0.01$ vs. MDM Surface Expression). (B) MDM uptake of fluorescent Zymosan (125 μ g/ml, 20min) or (C) *E. Coli* (1mg/ml, 1h) particles upon MCs application. Soluble α 2MG (94ng) was used for comparative purposes. Data are mean \pm SEM of 4 different human donors. One way ANOVA, Bonferroni post-test, $**p<0.01$ vs. vehicle, $^+p<0.05$ vs. 1×10^5 α 2MG-MCs. (D) Representative confocal Z-stack images of MDM incubated with *E.Coli* alone (vehicle) (left panel), α 2MG-MCs and *E.Coli* (central panel) or empty MCs and *E.Coli* (right panel) as analyzed after phagocytosis. (E) Representative light microscopy images of MDM after 24h incubation with MCs (1×10^5 cells with 1×10^5 MCs).

α 2MG-MCs have protective effects on a model of peritonitis.

In the final part of the study we queried whether α 2MG-MCs could be active during the complex and integrated settings of an inflammatory reaction. To parallel the *in vitro* data, we focused on neutrophil recruitment and macrophage phagocytosis. In a model of peritonitis, intravenous administration of α 2MG-MCs (selected dose of 1×10^5 /mouse) augmented the extent of neutrophil recruitment to the site of inflammation: approximate +60-80% increment in cell numbers over empty microcapsules or soluble α 2MG (94 ng/mouse; Figure 5A).

In line with the profiles of acute peritonitis [18, 19] neutrophil numbers and not macrophage levels were affected by the α 2MG-MC treatment, as demonstrated by the use of cell-specific markers (Gr1 for granulocytes and F4/80 for macrophages) (Figure 5A). Next, α 2MG-MCs were tested for their ability to augment macrophages phagocytosis.

Thus, macrophages were elicited into the peritoneal cavity prior to treatment i.p. with α 2MG-microcapsules (1×10^6 /mouse), empty-microcapsules (1×10^6 /mouse), soluble α 2MG (940ng/mouse) or vehicle (PBS). The uptake of fluorescently labeled Zymosan A was assessed 18 h later, using a 30 min time point. Flow cytometry analysis allowed quantification of the cells engulfed with fluorescent particles, as presented in Figure 5B. Mice treated with α 2MG-MCs displayed an increased number of Zymosan^{+ve} macrophages compared to the control group (>70%, $p<0.01$) and to soluble protein (>50%, $p<0.05$). These results were further corroborated in the *ex-vivo* setting with *E.Coli* particles, highlighting a critical different pharmacology as compared with the soluble active protein (>30%, Supplementary Figure 4).

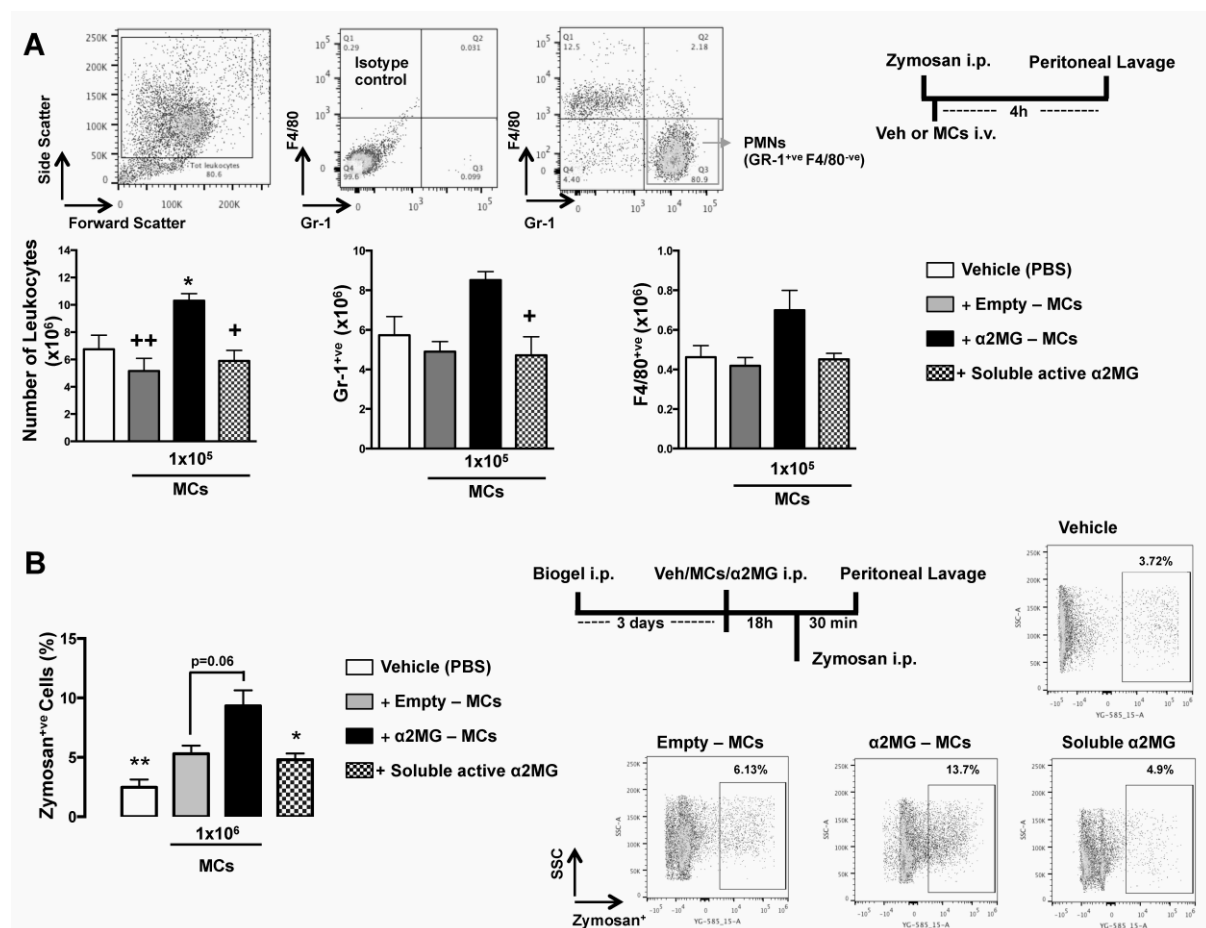


Figure 5. α 2MG-MCs promote extravasation and phagocytosis within the peritoneal cavity. (A) Vehicle (PBS), empty MCs (1×10^5 /mouse), α 2MG-MCs (1×10^5 /mouse) or α 2MG (94ng/mouse) were administered i.v. followed by zymosan A (0.1 mg i.p.). Leukocyte infiltration was assessed in peritoneal lavages by differential flow cytometry using anti-Gr-1 and anti-F4/80. Example of gate strategy for Gr-1⁺/F4/80⁺ neutrophils is showed in the upper panel. Data are M \pm SEM of 5 mice/group (*p<0.05 vs. vehicle, +p<0.05 vs. 1×10^5 α 2MG-MCs). (B) Vehicle, empty MCs and α 2MG-MCs (1×10^6 /mouse), or soluble active α 2MG (940ng/mouse) were injected i.p. into mice which had received 3 days earlier an injection of Biogel. After 18h, fluorescent Zymosan A was injected and 30 min later presence of engulfed particles in macrophages was evaluated by flow cytometry. Data report the percentage of macrophages positive for Zymosan particles (exemplars plots are on the right hand side). Data are mean \pm SEM of 4 mice/group (Unpaired t-test, *p<0.05, **p<0.01 vs. α 2MG-MCs).

Finally to establish downstream mechanisms potentially responsible for the activating properties of α 2MG-MCs on the acute inflammatory response, metabololipidomics was performed on cell-free peritoneal lavages. As depicted in Figure 6, mice treated with α 2MG-MCs, in comparison to empty-MCs, displayed higher levels of the pro-inflammatory chemoattractant LTB₄, which derives from the lipoxygenation of arachidonic acid. The concentrations of the pro-resolving PDX were also augmented post-treatment with α 2MG-MCs as compared to empty-MCs. Figure 6A shows the chromatographic profiles for PDX, LTB₄ and the cyclo-oxygenase product PGE₂, and their cumulative values: it can be seen that the effects were not univocal and, for instance, levels of PGE₂ were not altered by

microcapsule administration. Figure 6B shows the typical fragmentation patterns for these exemplar mediators following mass spectrometry. Moreover, the precursors for LXA₄ and resolvins/maresins were augmented upon administration of the α 2MG-MCs (Figure 6C). Vehicle (PBS)-generated mediators were comparable to levels measured after treatment of mice with empty-MCs (not shown).

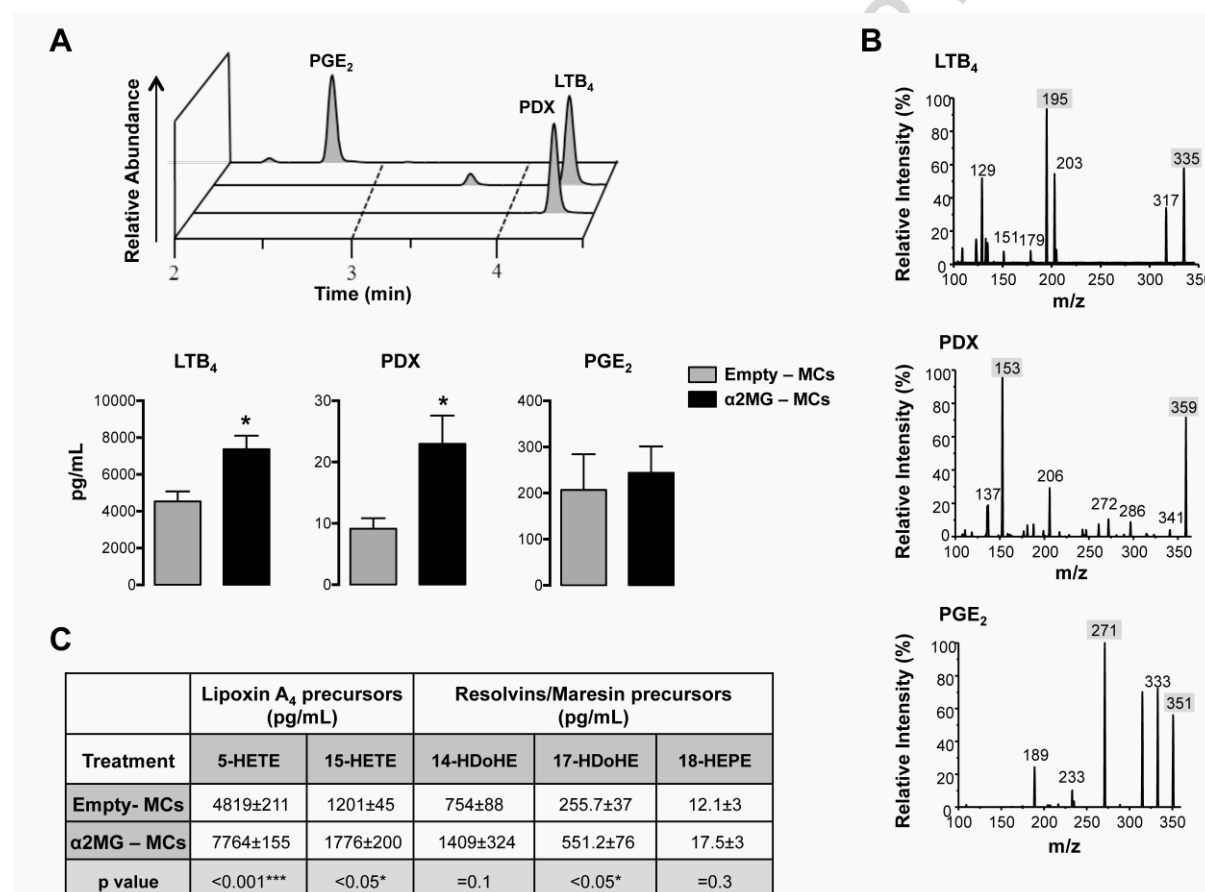


Figure 6. α 2MG-MCs increase pro-resolving lipid mediators. Mice were subjected to the phagocytic assay as in Figure 5B. Lipids of interest were determined in peritoneal lavage by LC-MS/MS. (A) Upper panel: Spectrum of relative peaks for Protectin DX, LTB₄ and PGE₂. Lower panel: LTB₄, Protectin DX and PGE₂ relative bars graphs. (B) Protectin DX, LTB₄ and PGE₂ signature fragment ions reported as m/z, highlighting the diagnostic fragments. (C) 5-HETE, 15-HETE, 14-HDoHE, 17-HDoHE, 18-HEPE were identified and quantified by LC-MS/MS and divided according to their downstream lipid product. Data are mean \pm SEM of 4 mice/group (Student's *t* test, *p<0.05 vs. empty-MCs).

DISCUSSION

We report here the generation of microcapsules loaded with α 2MG and describe their biological efficacy on the modulation of human endothelial cell and macrophage reactivity. These analyses are completed with proof-of-concept experiments in the mouse that reveal the ability of α 2MG – when delivered by the microcapsules – to incite the innate immune response, a mechanism that could underlie the reported efficacy in a model of sepsis [9]. Collectively these sets of experiments demonstrated that α 2MG-MCs retain immune-

modulatory properties during complex inflammatory settings, promoting neutrophil migration during acute inflammation and augmenting the phagocytic properties of macrophages.

Natural vesicles are emerging as a novel means for cell-to-cell communication with potential impact in physiology as well as pathology, which would include valid use as biomarkers [20] and indeed therapeutic tools [21]. However, these vesicles contain a large number of proteins and other cargoes, e.g. microRNA and lipids, which could limit their application or their efficacy over prolonged administration. For instance, an unwanted antibody reaction to specific proteins present in the vesicles would be detrimental for therapeutic development, even more so if the antigenic protein(s) result to be one that is not pivotal to the biological properties of interest. To this end, the exploding field of nanomedicines could come in succor allowing encapsulation of specific bioactive molecules and ameliorate their targeting or pharmacokinetics, in all cases impacting on the pharmacodynamics (e.g. see recent examples for an anti-inflammatory peptide [22, 23]). Herein we focused our attention onto α 2MG since we demonstrated i) presence of this protein in neutrophil-derived natural vesicles [8], ii) its segregated expression in vesicles quantified in patients surviving sepsis [9] and iii) its ability to modulate mouse cell reactivity, leading to partial protection in experimental sepsis [9].

Efficient encapsulation of α 2MG into microcapsules was achieved, obtaining synthetic structures with the expected physical characteristics of shape, size and stability. This extends α 2MG to the list of biologically active tools that can be entrapped in these dextran-based microstructures [5, 24]. In line with recent studies, the efficiency of encapsulation was high with a limited loss of protein during the process, calculated to be less than 1%. Encouraged by these results, then we tested α 2MG-MCs in experimental conditions using human cells central to the immune response, these being endothelial cells and macrophages. Crucial comparisons were made with empty MCs, prepared alongside identical protocols, and/or soluble α 2MG: our prediction was that α 2MG-MCs could harness α 2MG biology with higher efficacy over the soluble protein, presenting also specific biological properties when compared to the empty microcapsules. In human umbilical vein endothelial cells, α 2MG-MCs led to increased adhesion of human neutrophils. This process is a hallmark of inflammation: neutrophils are the first cell type to reach the site of injury and in order to do so must initially interact with the vessel wall [25]. Using confocal studies, we could unveil an effective interaction and penetrance of the microcapsules into the endothelium with consequent exposure of active α 2MG. A background level of α 2MG was detected onto the monolayer, even in the absence of addition of α 2MG-MCs: this is plausibly due to the presence of the protein in the plasma added to the cell culture. In any case, we predicted that the active protein presented on the surface of endothelial cells would then interact with its receptor LRP1 presented by the rolling neutrophil, promoting the subsequent step of adhesion. LRP1 was initially described as a 'silent' endocytic receptor [26], but evidence is emerging that it can signal upon application of α 2MG [27, 28]. The model that emerges is one where α 2MG presented by the endothelial monolayer activated the flowing neutrophil promoting the transition from rolling to adhesion, thus committing to extravasation.

Meanwhile further studies are required to address the molecular events evoked by α 2MG engagement of LRP1 on the rolling neutrophil, it is noteworthy that the model presented herein held consistency *in vivo*. Intravenous injection of α 2MG-microcapsules – at doses

coherent with what used *in vitro* when one considers an approximate total blood volume of 3-5ml in the mouse – augmented neutrophil extravasation during on-going peritonitis. Such an effect would be beneficial on the way the host could combat an infective status, as discussed below. Intravenous injection of 1×10^5 capsules confirmed their inability to cause toxicity (all animals survived). Furthermore, a specific experiment was performed to localize the injected capsules in liver, spleen and lung. This was partially successful, since strong autofluorescence of liver and spleen hampered easy detection of the capsules (data not shown). In contrast, we could identify $\alpha 2$ MG microcapsules only in lung tissue sections by fluorescent microscopy (Supplementary Figure 5). However, we are confident that the capsules would have been taken up by liver and spleen, as demonstrated in a recent study using capsules with similar chemical composition [29]. Next, we focused our attention onto the human macrophages, central to initiation and resolution of the inflammatory response [30] and the main cell type devoted to phagocytosis.

Initial works with similar microcapsules have demonstrated uptake of the microstructures by immortalized macrophage cell lines, both from human and murine origin [31] however studies with primary cells are lacking. Addition of $\alpha 2$ MG-microcapsules onto human monocyte-derived macrophages resulted in a time-dependent uptake (Supplementary Figure 1B). At variance from the human endothelial cells, macrophage uptake was optimal over a longer time-point, with maximal result at 24 h incubation. However, this kinetics is similar to that observed for the empty microcapsules and it is, therefore, a feature of these microstructures and not secondary to the physical presence of $\alpha 2$ MG or one of its biological effect. Interestingly, only a subset of macrophages was shown to take up the microcapsules. This was a consistent result with a proportion of cells engulfing well over one or two microcapsules and other adjacent cells being empty, with no microcapsules at all. The molecular explanation of this dichotomy of response is unclear however it is not uncommon and previously observed for other phagocytic stimuli [17].

Once inside the human macrophage, $\alpha 2$ MG was in part released by the microcapsules and consistently promoted phagocytosis of other stimuli, as shown with two unrelated particles of pathogenic relevance, Zymosan A that is a major component of the yeast wall and the Gram negative bacterium *E. coli*. In line with the kinetics of the phagocytosis process (e.g. see our work with mouse macrophages and endogenous Annexin A1 [32]), the engulfment of these pathogenic particles was rapid with significant values within 30-45 min, yet augmented by previous uptake of the $\alpha 2$ MG-MCs, as demonstrated by two experimental approaches using different preparations of *E. Coli* (Figure 4C and Supplementary Figure 2). It is yet unknown how the $\alpha 2$ MG-MCs would accelerate this response of the macrophage and this will require further studies. The cytoplasmic domain of LRP1 contains the necessary structural features to mediate phagocytosis [33]; it is unclear whether $\alpha 2$ MG would interact with LRP1 onto the phago-lysosome, inside the macrophage, or if the protein is externalized to interact with LRP1 in a paracrine or juxtacrine fashion.

The experiments with human monocytes and MDM provided another interesting observation: LRP1 was present in the former though increased expression was quantified upon differentiation in the latter, reflecting the phagocytic role of macrophages [34]. We concluded

this set of experiments by reasoning again that it was important to establish whether the positive modulation of macrophage phagocytosis could be replicated *in vivo*. To this end, intraperitoneal injection of α 2MG-MCs promoted the uptake of fluorescent Zymosan and increased lipid mediators' levels. The metabololipidomics revealed interesting mechanistic connections. Whilst the augmented level of LTB₄ in α 2MG-MC-treated mice is in accordance with the enhanced extent of neutrophil recruitment we showed in peritonitis, the elevation of PDX was of particular interest. This bioactive compound is produced via sequential lipoxygenation of docosahexaenoic acid (22:6 *n*-3) and can be found together with the first identified protectin, protectin D1 (PD1) in mouse inflammatory exudates [35]. Moreover, PDX and the other bioactive tissue-protective mediators including the LXA₄ and Resolvins or Maresins are potent inducers of macrophage phagocytosis [36, 37]. It is of further interest that α 2MG-MCs afforded significant modulation of lipoxygenase dependent metabolism with little or no detectable effect on the cyclo-oxygenase pathways.

Altogether these new results complement well the macroscopic amelioration of experimental sepsis [9, 38]. Our new data with human cells and the mechanism revealed herein indicate that α 2MG-MCs could be developed for the management of human sepsis. From a mechanistic perspective, efficacy in septic settings would derive from an augmentation of neutrophil extravasation and macrophage phagocytosis, cellular event of paramount importance in the way the host would deal with the bacterial attack. A more general implication entails the appreciation that synthetic microcapsules, filled with key players of the inflammatory process, can harness their tissue-protective biology by prolonging stability in the organism, and ultimately represent a novel strategy for the delivery of therapeutics.

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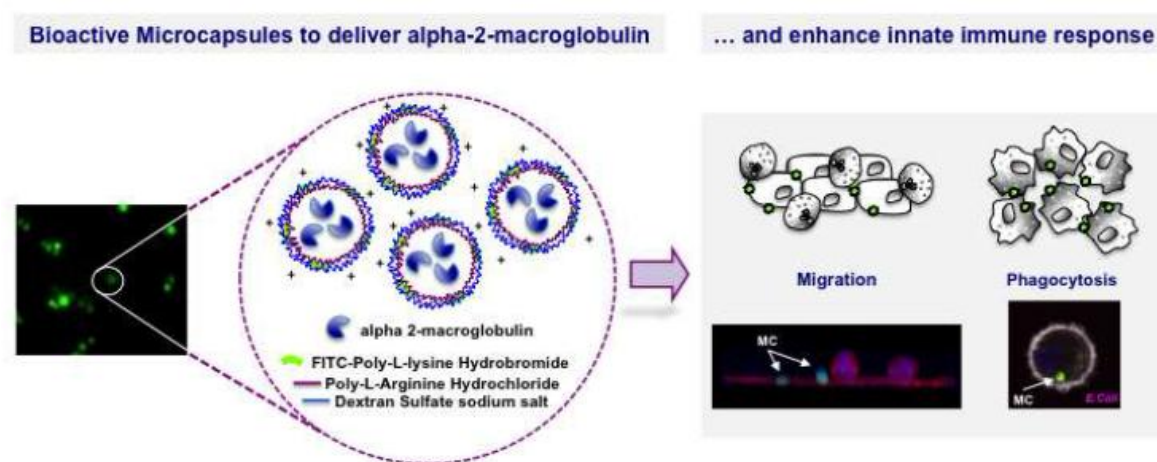
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Graphical abstract