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NOVEL ENRICHED MICROCAPSULES TO DELIVER INFLAMMATION-MODULATING PROTEIN: THE EXEMPLAR OF α2-MACROGLOBULIN

XXV Cycle PhD Program in Molecular Medicine

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INTRODUCTION

Microparticles (MPs) are a heterogeneous population of small membrane-bound vesicles released from different cell types. They were first reported in 1967 in human plasma as platelet derived-small fragments (Wolf P, 1967) and, for a long time, considered residues of platelet activation or cells debris. Subsequent studies revealed that microparticles are actually subcellular elements that serve as important signalling structures between cells, and that they can be actively involved in physiological and patho-physiological processes, representing a storage pool of bioactive effectors (VanWijk *et al*., 2003). Besides platelet, microparticles can be generated from several cell types, including endothelial cells, neutrophils, monocytes, macrophages, B- and T-cells and erythrocytes. Importantly, microparticles present cell surface markers and cytoplasmatic components of their precursor cells (Distler *et al*., 2005; Dalli *et al*., 2008). Depending on their origin, microparticles differ in size (diameter 0.1-1 μm), protein and lipid composition, and, as a consequence, they can elicit distinct effects. Indeed, against their initial description as cellular debris, it has become clear that microparticles are novel subcellular effectors that can regulate important cellular processes, e.g. thrombosis, vascular reactivity and inflammation (Berckmans *et al*., 2001; Ardoin *et al*., 2007).

How are microparticles produced? There are two well-known cellular processes that can lead to the formation of microparticles: cell activation and apoptosis (VanWijk *et al*., 2003). Current wisdom is that microparticles differ according to whether they are produced by either process, both in term of macromolecular composition, both external and cytoplasmatic (Jimenez *et al*., 2003; Bernimoulin *et al*., 2009). This suggests that microparticles may not be formed through a single process, but rather that the release of microparticles differs qualitatively and quantitatively, depending on parent cell types as well as on the extent of process of apoptosis.

Recent studies on inflammation, have focused on microparticles derived from phagocytes (mainly neutrophils and monocytes), and reported different, and in some cases conflicting, results. In fact, in relation to the type of generating stimulus, microparticles can exert pro- or anti-inflammatory effects (Freyssinet, 2003). For example, neutrophil-derived microparticles induce the expression of IL-6 and monocytes chemotactic protein-1 (MCP-1) in endothelial cells (Mersi and Altieri, 1999) but, under different circumstances, they exert anti-inflammatory effects, including production of TGF-β1, inhibition of the release of IL-8, IL-10 and TNF-α in macrophages (Gasser and Schifferli, 2004). They can also inhibit cell recruitment and adhesion (Dalli *et al*. 2008). Additionally, monocyte-derived microparticles, can induce pro-inflammatory effects in human monocyte/macrophages, but can also enhance Perixosome Proliferation-Activated Receptor-gamma (PPARγ) protein expression in the same cells, representative of an anti-inflammatory

response (Bardelli *et al*., 2012). Unpublished work in my lab suggests a possible explanation for this dichotomy insofar as neutrophil-derived microparticles were shown not only to contain ~400 proteins, hence a large amount of possible mediators, but also to have partial distinct proteome in relation to the stimulus applied. Therefore, presence of distinctive proteins – reflecting the status of the cell and/or the environment where it is located – could explain the apparent discrepancy in biological functions ascribed to a specific set of microparticles even when produced from the same cell type.

The multiple roles of microparticles and their functional importance during inflammation are, therefore, increasingly valued and their potential relevance as endogenous controllers of the inflammatory process is of considerable interest. Moreover, it is now plausible to take advantage of their features through the creation of specific microparticles with well-defined cargo. Several studies in last decade have been focused on the construction of nano- and micro- capsules engineered as carriers for active compounds, for example enzymes, nucleic acids, proteins, chemo-therapeutics and drugs in general. Briefly, the method is based on layer-by-layer assembly of polymers to make micro- and nano-capsules with defined size, composition and content of encapsulated materials. Shell thickness is determined by the number of layers and can differ from 50 nm to 10 μm, and the core of the particles can be dissolved and refilled with substances of interest (Antipov and Sukhorukov, 2004; Sukhorukov and Möhwald , 2007). The group of Sukhorukov, at School of Engineering and Materials Science (Queen Mary University of London), has established a new technology based on sequential deposition of 7 polymer layers: poly- L-Arginine, dextran sulfate sodium salt and poly- L-Lysine to allow the construction of microcapsules (Sukhorukov GB and Möhwald H, 2007; She *et al*., 2010). These capsules are stable at 37°, do not produce any toxicity when added to cells and can be engineered to facilitate internalization by target cells (De Cock *et al*., 2010).
This **capsules technology**' allows mimicking the endogenous microparticles but can also facilitate the study of the effects of a single encapsulated mediator on the process of interest and on specific target cells. Moreover, it can represent a unique system for packaging, storing and delivering bioactive molecules, as well as regulating biological function.

Among the several mediators of the inflammatory process discovered in microparticles, **alpha-2-macroglobulin** (α2M) is of particular interest. Indeed, α2M is a tetrameric plasma protein (composed of four identical 180 kDa subunits) and is one of the acute phase proteins, evolutionarily conserved and expressed in a variety of cell types.

α2M acts as a protease inhibitor (e.g. for plasmin and thrombin) and can also be a carrier for several growth factors and cytokines, as platelet-derived growth factor (PDGF), TNF-α, IL-1β, IL-6 and TGF-β (Borth, 1992; Feinman, 1994). The native state of α2M contains a surface-exposed

peptide (the “bait region”) representing a recognition site for multiple proteases. The cleavage of the “bait region” leads to activation of α2M that involves a complex conformational change of the tetramer and can be triggered either by protease cleavage of α2M or by methylamine treatment. Activation of α2M results in the entrapment of proteases, and the entire complex binds to the α2M receptors, nowadays termed low density lipoprotein receptor like protein-1 (LRP1; Andersen *et al*., 2000). The protease–α2M-receptor complex is then internalized and can undergo lysosomal degradation, with the receptor being eventually recycled to the cell surface (Borth, 1992). LRP-1 is a member of LDL receptor gene family and it is a ubiquitously expressed type 1 transmembrane receptor. LRP-1 can recognize at least 40 different ligands, it is endowed with scavenger properties and it now seems, can also signal; it is no surprise then that this receptor is being implicated in numerous process. For example, studies have shown its vascular-protective effects during the development of atherosclerosis, modulation of the progression of Alzheimer’s disease by binding β-amyloid precursor protein (APP) and also its involvement in macrophages phagocytosis process (Nilsson *et al*., 2012; Fadok *et al*. 2001; Boucher and Herz, 2011; Lillis *et al*., 2008; Herz and Strickland, 2001).

α2M –LRP1 axis has a great potential to the regulation of cytokine homeostasis in blood and tissue. Indeed, the mechanism of action of α2M that bind and neutralize some inflammatory cytokines is thought to be essential in certain condition, including sepsis (Birkenmeier *et al*., 2006; Webb and Gonias 1998).

In unpublished work, that forms the foundation of the current project, α2M has been found to be abundant in a specific subset of neutrophil derived microparticles, and to be a major determinant for their protective effects in sepsis. The presence of this plasma protein in microparticles could be a consequence of its uptake through pinocytosis by neutrophils and incorporation into secretory vesicles (Borregaard *et al*., 1992). Sepsis is characterized by an immune paralysis phase, with neutrophils dysfunction, leading to predominance of an anti-inflammatory status (compensatory anti-inflammatory syndrome-CARS) (Goldenberg *et al*., 2011). Neutrophil-derived microparticles positive for α2M were significantly abundant in the plasma of sepsis-survivor patients compare to non-survivor and healthy volunteers. Additionally, these microparticles enriched with α2M acted as nanomedicines with potent organ protective properties in a model of murine sepsis, where they reduced bacterial load in peritoneal exudates and blood, decrease neutrophil numbers and their infiltration into the lungs (Dalli *et al*., submitted). α2M has powerful stimulatory effects on neutrophil responses, in fact, in the same study, they demonstrated that exposure of endothelium to α2M-enriched microparticles can increase the extent of neutrophil adhesion.

Since α2M is one the determinant of microparticles and play a crucial role in severe pathologies, including sepsis, this project aim at exploiting the capsules technology to recapitulated

the biological function of this protein in synthetic structures, in order to mimic the endogenous microparticles and study the effects of α2M on the process of interest and on specific target cells. The rationale was to study these effects in the absence of the other proteins present in the natural microparticles. Furthermore, this can represent an important step in manufacturing microcapsules enriched with biologically relevant proteins hence with potential therapeutic/delivery application.

To do so, I spent the third year of my PhD program (Molecular Medicine) in the Centre for Biochemical Pharmacology, William Harvey research Institute, Queen Mary University of London and I was involved in the first characterization of novel enriched microcapsules to deliver inflammation-modulating proteins. In particular, the aim of my study was to evaluate the biological property of the newly generated α2M -microcapsules in a *in vitro* assay of neutrophil-endothelial cell interaction (flow chamber) and in phagocytosis assay using human monocyte-derived macrophages (MDM), isolated from peripheral blood of healthy volunteers.

METHODS

Generation and characterization of α2m enriched-microcapsules

α2m enriched-microcapsules were generated by the group of Sukhorukov (SEMS) along their established procedures (Sukhorukov and Möhwald, 2007). Briefly, the capsules were generated by alternating deposition of 7 polymer layers: poly- L-Arginine, dextran sulfate sodium salt and fluorescent FITC-poly- L-Lysine (positive outer charge) that constitute the shell of microcapsules. These layers are deposited around a core of $CaCO_3$ that, at the end of the process, is dissolved using 0.2M ethylenediaminetetra-acetic acid (EDTA). One milligram of active α2M was incorporated into the microcapsules. As a control, a blank preparation of microcapsules was used, prepared and handled exactly as the α2M-microcapsules but devoid of any protein.

α2m-microcapsules and blank-microcapsules were first counted with a haemocytometer and controlled by flow cytometry using the forward and side scatter analysis, together with 1μm beads for comparison. Their content of α2m was then assessed by Western Blot analysis: 20μl of α2m-microcapsules and blank-microcapsules solution were added to 8% acrylamide gel and electro-blotted on PDVF membrane. The membrane was incubated overnight with specific α2m antibody (1μg/ml in 5% milk; clone 257316, R&D System) and then detected with anti-mouse secondary antibody coupled to horseradish peroxidase (Dako). Chemiluminescence signals were analysed under non-saturating conditions with an image densitometer (FluorChem E, ProteinSimple, Santa Clara, USA).

Both the microcapsules preparations confirmed the expected characteristics in terms of size and content.

Preparation of monocytes, monocyte-derived macrophages (MDM) and neutrophils

Human neutrophils (PMNs) and monocytes were isolated from venous blood (50 ml) of healthy volunteers that was collected into 3.2% sodium citrate, centrifuged at 800 rpm for 20 min at room temperature. PRP was removed and cells were left to sediment by adding 6% Dextran (Sigma) in Dulbecco phosphate-buffered saline (DPBS), for at least 15 min to sediment red blood cells. The upper layer was then added to Histopaque (density = 1.077 g cm^{-3}) and centrifuged at 1500 rpm for 30 min at room temperature.

After gradient centrifugation, monocytes and neutrophils were both collected. The monocyte-enriched layer was recovered by thin suction at the interface, as described (Amoruso *et al*., 2010, 2009). Cells were washed twice in DPBS and finally re-suspended in RPMI 1640 medium, supplemented with L-glutamine (2 mM), Hepes (10 mM), 50 µg/ml streptomycin and 5 U/ml penicillin. Purified monocyte populations were obtained by adhesion (1 h, 37°C, 5% CO_2), non-adherent cells (mainly lymphocytes) being gently removed with sterile DPBS. Monocytes were then cultured in RPMI 1640 medium supplemented with 5% heat-inactivated foetal bovine serum (FBS). Monocyte-derived macrophages (MDM) were prepared from monocytes, by culture (8-10 days) in RPMI 1640 medium containing 20% FBS, glutamine and antibiotics (as above). This method ensures the differentiation of monocytes towards macrophage-like cells as previously described (Amoruso *et al*., 2007).

Immediately after removing the monocyte-enriched layer, the lower layer containing neutrophils was also collected. The contaminating few erythrocytes were removed by hypotonic lysis: cells were re-suspended in ice-cold water for a few seconds and then the osmotic balance was restored with Hanks' Balance Salt Solution (Sigma). Cells were then washed twice in DPBS without Ca^{2+} and Mg^{2+}, and left at 4˚C until experimentation.

Preparation of endothelial cells and in vitro flow chamber assay

Umbilical cords were supplied by the midwifery staff of the maternity unit, Royal London Hospital. Primary human umbilical vein endothelial cells (HUVEC) were isolated by collagenase digestion of the interior of the umbilical vein (Jaffe *et al*., 1973) and collected in T75 flasks in complete medium (M199) supplemented with penicillin (100U), streptomycin (100mg/ml), fungizone (2.5 µg/ml), L-glutamine (2mM) and 20% of human serum (HS, Lonza). HUVEC were used at passage 2 for the experiments.

To assess leucocyte-endothelial interaction, HUVEC were plated in µ-Slides $VI^{0.4}$ (Ibidi, Munchen, Germany), and confluent monolayers were stimulated with TNF-α (10ng/ml) (to up-regulate adhesion molecules such as E-selectin, ICAM-1 and VCAM-1) in presence or absence of different amounts of α2m-microcapsules or blank-microcapsules for 4h, at 37°C. In another set of flow experiments, HUVEC were incubated with active α2m (10nM, 4h) prior to flow and neutrophils adhesion was assessed. Immediately prior to flow, freshly prepared neutrophils were suspended at 1×10^6/ml in DPBS supplemented with Ca^{2+} and Mg^{2+} containing 0.1% bovine serum albumin (BSA) and incubated for 10 min at 37˚C. The entire flow chamber was placed under a Nikon Eclipse TE3000 microscope fitted with a x20 phase contrast objective (Nikon). Neutrophils were perfused over the monolayer at 1 dyne/cm^2 using a programmable syringe pump (Stoelting, Germany) for 8 min, and then 6 random fields/treatment were recorded for 10 seconds each.

Sequences were loaded into ImagePro-Plus software (Media Cybernetics, Wokingham), neutrophils were tagged and their migration monitored. The total number of interacting neutrophils was quantified as captured and further classified as rolling or adherent if stationary for the 10 sec period (Norling *et al*., 2012).

Confocal microscopy analysis

In order to visualize the microcapsules and cell interactions, after flow chamber assay, HUVEC and PMNs were immediately fixed with 1% paraformaldehyde (PFA) for 10 min at 4˚C and then washed with PBS. Cells were stained with Alexa Fluor® 633-Agglutinin (1μg/ml; Invitrogen) for 20 min at room temperature in the dark and then blocked for 30 min in PBS containing 5% FBS. Unconjugated primary anti-α2m antibody (5μg/ml; R&D System) was then added for 1h followed by Alexa Fluor® 594 secondary antibody (Invitrogen) for 45 min. Cells were finally washed in PBS and left in Probing Antifade medium (Invitrogen) containing DAPI. They were visualized using a Zeiss LSM 510 META scanning confocal microscope (x63 oil-immersion objectives) and analysed by Zeiss LSM Imaging software (Carl Zeiss, Oberkochen, Germany). The number of α2m-positive particles were acquired and counted in each sample using ImageJ software.

Flow-cytometric analysis

Monocytes and monocyte-derived macrophages (MDM) were assessed for both their surface and intracellular expression of α2m receptor (CD91) along with the lineage specific lineage marker (CD14 for monocytes, CD68 for MDM). Cells were washed with ice-cold PBS and scraped in ice-cold PBS containing 1% FBS. To analyse the intracellular expression cells were incubated with Permeabilization Buffer containing saponin (eBioscence) following the manufacturer's instructions. Both un-permealized and permealized cells were labelled with anti-CD14 APC-conjugated antibody for monocytes (0.5μg/ml; clone 61D3, eBioscence) and anti-CD68 PE-conjugated antibody (0.5μg/ml; clone Y1/82A, eBioscence) for MDM together with anti-CD91 FITC-conjugated antibody (5μg/ml; clone A2Mr alpha-2, AbD Serotec) antibodies and with the relevant isotype controls, for 1h at 4˚ in the dark. Cells were then washed with PBS and re-suspended in PBS containing 0.5% FBS prior to analysis with a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA) using CellQuestTM software (Becton Dickinson).

Zymosan and E.Coli preparation and Phagocytosis assay

MDM were evaluated for their ability to phagocytes Zymosan and Escherichia Coli (E. Coli) particles. MDM were seeded in black 96-well plates at a density of $2x10^5$ cells per well. Cells were first washed in sterile DPBS and then incubated in RPMI containing 0.1% FBS with different amounts of α2M-microcapsules and blank microcapsules for 24h in a final volume of 200 μl (at 37°, 5% CO_2 atmosphere).

Zymosan (Zymosan A, from Saccharomyces cerevisiae, Sigma) and E. Coli particles (Strain K12, Sigma) were conjugated with a fluorescent dye (Bodipy® 576/589, 1μM final concentration; Invitrogen) for 5 min at room temperature and unlabelled particles were washed by centrifugation in PBS at 1200 rpm for 10 min. Before using, both the preparation of labelled Zymosan and E.Coli particles were analysed by flow cytometry in order to confirm their conjugation.

After 24h of incubation with microcapsules, Zymosan was added to the medium at a final concentration of 125 µg/ml and cells were incubated for a further 20 min (at 37°C, 5% CO_2 atmosphere). In another set of experiments, E. Coli particles were added at a final concentration of 1 mg/ml and MDM were incubated for a further 60 min (at 37°C, 5% CO_2 atmosphere). In order to subtract the auto-fluorescent value, in each experiment, a control without fluorescent particles was included.

At the end of the incubation, cells were put on ice and wash 3 times with ice-cold PBS in order to remove non-phagocytosed particles. The number of fluorescent phagocytosed particles was determined with a fluorescence plate reader (NOVOstar, BMG LABTECH) and analysed using MARS Data Analysis Software (version 1.20 R2, NOVOstar, BMG LABTECH).

Statistical analysis

All statistical analyses were performed using GraphPad Prism (version 5.01 for Windows, San Diego California, USA). Data are expressed as mean ± SEM of "*n*" independent experiments on cells isolated from different healthy donors. Statistical evaluation was performed by unpaired two-tailed Student's t-test. Differences were considered statistically significant when $p < 0.05$.

RESULTS

Generation and characterization of α2m enriched-microcapsules

Blank and α2M-microcapsules were first counted with a haemocytometer, showing a total number of 850x10^6 of α2M-microcapsules and 528x10^6 of blank-microcapsules. They were then controlled by microscopy (**Fig. 1A**) and flow cytometry (**Fig. 1B**) in comparison with 1μm fluorescent beads: their size results to be approximately between 1-4 μm, as the endogenous microparticles are. Moreover, microcapsules were checked for their α2M content by Western Blot using a specific anti- α2M antibody (**Fig. 1C**), confirming the expected characteristics.

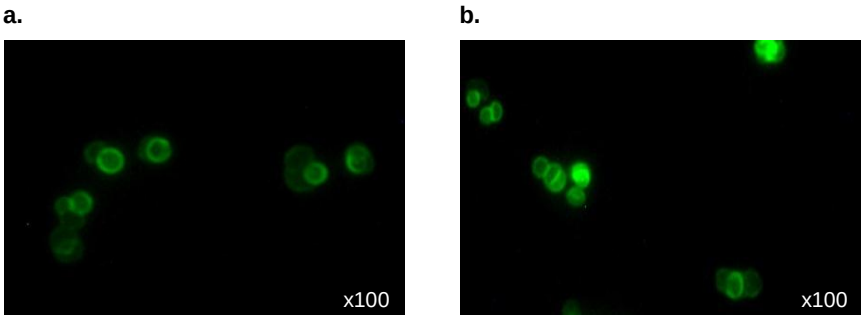


Fig.1A ***Fluorescent microscope analysis of microcapsules.*** α2M-microcapsules (a.) and blank-microcapsules (b.) were visualized under fluorescent microscope thanks to their conjugation with FITC probe.

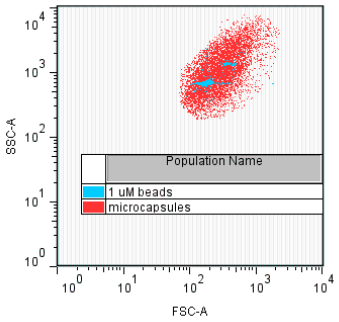


Fig.1B ***FACS analysis of microcapsules.*** α2M-microcapsules (red dots) were controlled by flow cytometry using the forward and side scatter parameters, together with 1μm beads for comparison (blue dots).

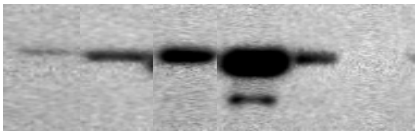


Fig.1C ***Western blot analysis of microcapsules*****.** α2M-microcapsules and blank-microcapsules were loaded into WB gel and incubated with anti-α2m antibody. Lane 1-4: free α2m 0.1-0.5-1-5 μg; lane 5: α2M-microcapsules; lane 6: blank-microcapsules.

α2M-microcapsules increase adhesion of neutrophil to endothelial monolayer in a *in vitro* flow chamber assay

α2M-microcapsules and blank microcapsules were tested in the flow chamber assay. It has been showed that α2M increases the interaction and adhesion of leucocytes to endothelial cells (submitted). Therefore, we wanted to verify if α2M-microcapsules could mimic this effect. Endothelial cells were stimulated with TNF-α (10ng/ml) for 4h in presence or absence of different amounts of α2m-microcapsules or blank-microcapsules, from $0.01x10^5$ microcapsules to $1x10^5$. Human freshly isolated neutrophils were then flowed over the monolayer and the number of captured and adherent neutrophils was acquired.

In our experiments, stimulation of endothelial cells with α2M-microcapsules significantly increases the recruitment and adhesion of neutrophils to endothelial monolayer, in a concentration-dependent manner (*p<0.05 *vs.* control: TNFα stimulated endothelial cells) (**Fig. 2A**). Of note, α2m-microcapsules had similar effects of soluble active α2m (10nM) (**Fig. 2B**).

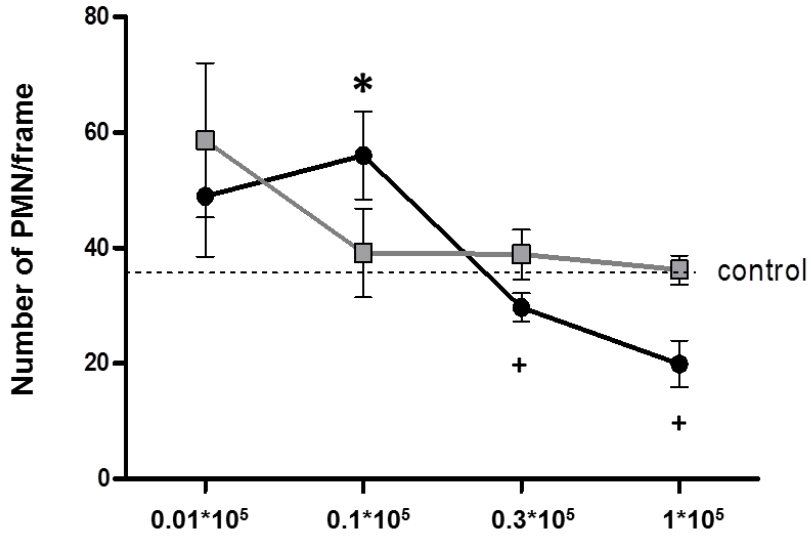


Fig. 2A ***Flow chamber assay*.** Endothelial cells were incubated with TNFα (10ng/ml) in presence or absence of α2m-microcapsules or blank microcapsules for 4h, at 37°. Then, freshly prepared neutrophils were perfused over the monolayer and the number of adherent neutrophils was quantified. The control level is represented by the dot line. Data are mean ± SEM of 4-12 different HUVEC and neutrophils preparations (Unpaired t-test: *p<0.05 *vs.* control; +p<0.05 *vs.* α2m microcapsule $0.1*10^5$).

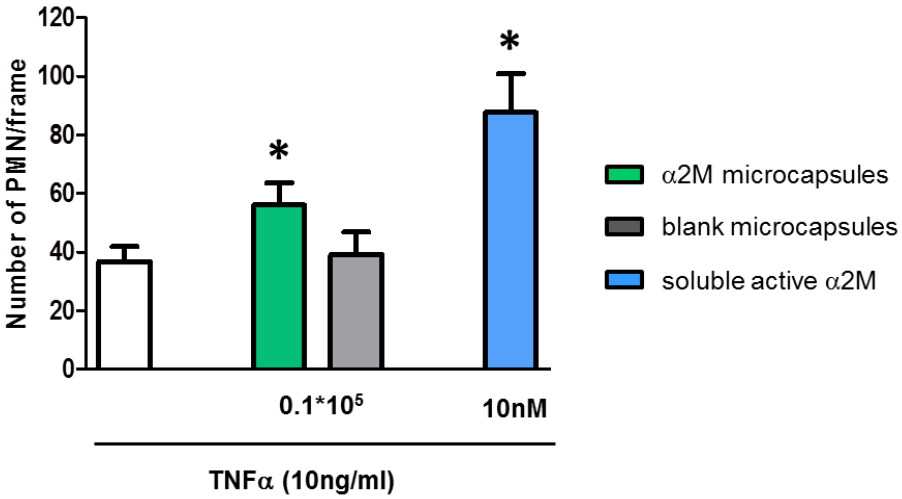
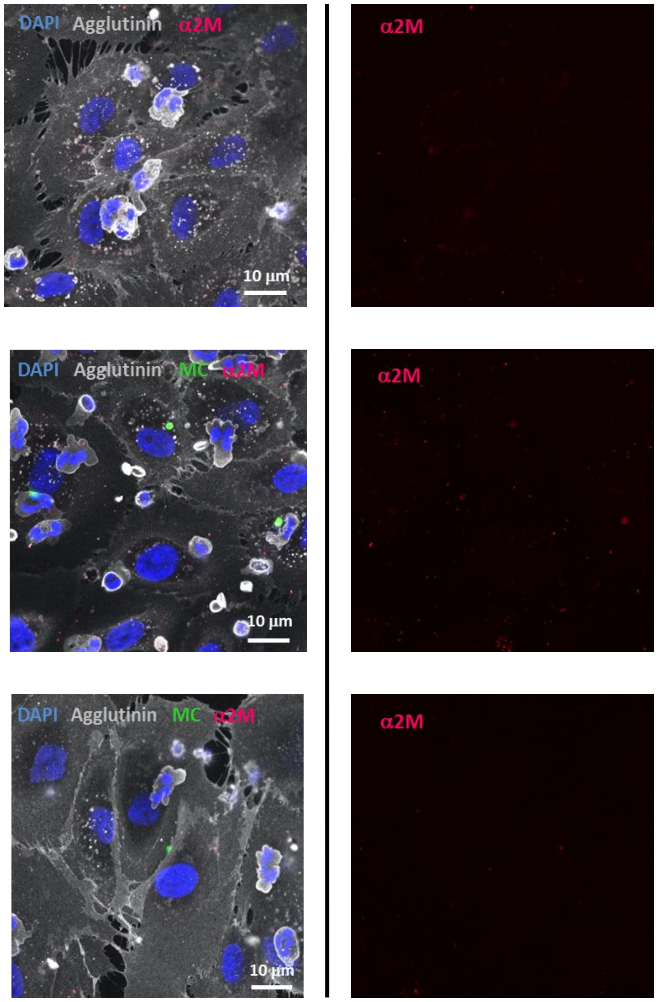


Fig. 2B ***Flow chamber assay***. The effects of microcapsules were compared to those of soluble active α2m used at 10nM. Endothelial cells were incubated with TNFα (10ng/ml) in presence or absence of α2m-microcapsules, blank microcapsules or soluble α2m for 4h, at 37°. Data are mean ± SEM of 4-12 different HUVEC and neutrophils preparations (Unpaired t-test: *p<0.05 *vs.* control).

α2M-microcapsules increase the expression of α2m on the surface of endothelial cells

After the flow experiments, monolayers of endothelial cells and neutrophils were stained with a specific anti-α2m antibody, along with fluorescent Agglutinin that selectively recognizes sialic acid and N-acetylglucosaminyl residues which are predominantly found on the plasma membrane (**Fig. 3A**). Confocal microscopy images demonstrate how incubation with α2M-microcapsules significantly increases the expression of α2M on the surface of endothelial cells. This effect was not replicate in the case of cell incubation with blank-microcapsules (**Fig. 3B**).



a. TNF-α stimulated endothelial cells

b. α2m-microcapsules stimulated endothelial cells

c. blank-microcapsules stimulated endothelial cells

Fig. 3A ***Confocal images after flow experiments***. After the flow chamber assay, cells were labelled with fluorescent Agglutinin (grey staining) and an anti-α2M antibody (red staining). On the right panel of each treatment (**a**. control; **b**. α2m-microcapsules; **c.** blank-microcapsules) it is showed just the staining with anti-α2M antibody, to underline the differences between them. Images are representative of 3 different flow chamber experiments.

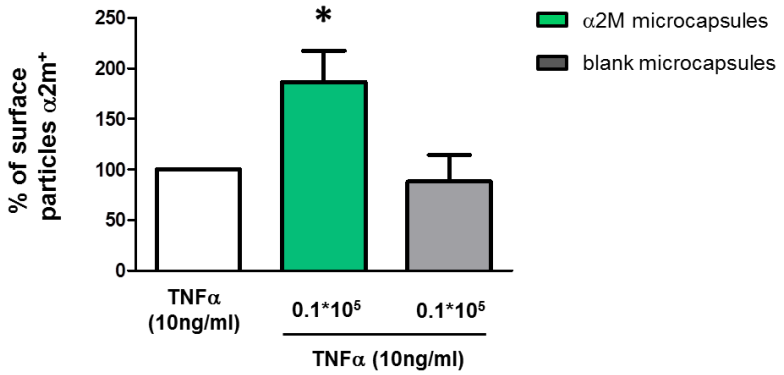


Fig. 3B ***Confocal images after flow experiments***. After the flow chamber assay, cells were labelled with anti-α2M antibody (1h at 4˚ in the dark) and the number of positive particles was acquired using ImageJ software and expressed as percentage over control. Data are mean ± SEM of 3 different flow chamber experiments (Unpaired t-test: *p<0.05 *vs*. control).

α2M-microcapsules increase macrophage phagocytosis

α2M-microcapsules have been also tested in 96-wells phagocytosis assay using human monocyte derived macrophages (MDM) isolated from peripheral blood. This assay was run because neutrophil-derived microparticles have been showed to augment macrophage phagocytosis (a response with a positive impact on the host response to sepsis and infection) (submitted).

The α2M specific receptor LRP1 (cluster definition CD91) is generally recognized as a macrophage receptor critical for the engulfment of apoptotic cells (Fadok *et al*., 2001). Therefore, we initially tested if LRP1 was present in monocytes and whatever there were differences in its expression during the differentiation process to MDM. Cells were analysed by FACS staining with an anti-CD91 antibody, along with the lineage specific marker: CD14 for monocytes (**Fig. 4A**) and CD68 for macrophages (**Fig. 4B**). Cell preparation was run untouched or following permeabilization with saponin, thus quantifying both cell surface and intracellular receptor expression (**Fig. 4A, 4B**). These data demonstrate that CD91 is expressed in monocytes predominantly on the cell surface, whereas, after differentiation in MDM, the receptor is mainly intracellular with low expression on the surface. This raises the hypothesis that during the differentiation, CD91 can be internalized and that resting macrophages (as these MDM are) despite having LRP1, they may express it on the surface just upon activation.

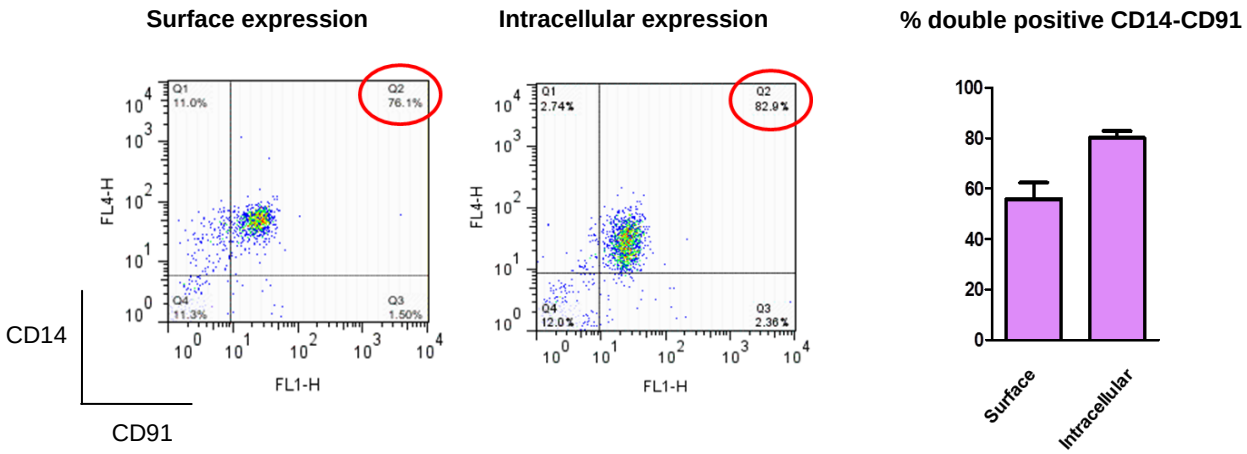


FIG 4A. ***Facs analysis of human monocytes*.** Cells were labeled with fluorescently conjugated anti-CD14 and anti-CD91 antibodies for 1h at 4˚ in the dark. Data are mean ± SEM of 4 different monocytes preparations.

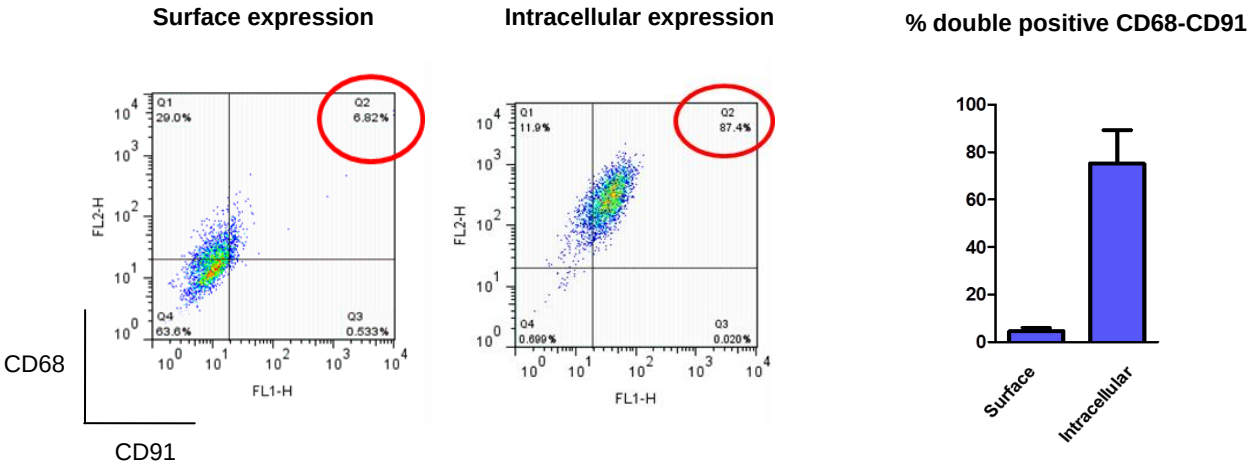


FIG 4B. ***Facs analysis of human MDM*.** Cells were labeled with fluorescently conjugated anti-CD68 and anti-CD91 antibodies for 1h at 4˚ in the dark. Data are mean ± SEM of 4 different macrophages preparations.

Once verified the expression of the receptor for α2m in MDM, cells were incubated with different amounts of α2m-microcapsules or blank-microcapsules for 24h. In a set of experiments, fluorescent conjugated Zymosan particles (**Fig. 5A**) were added to cells and phagocytosis was monitored. As depicted in **Fig. 5B**, incubation of MDM with α2M-microcapsules can significantly promote their ability to phagocytes Zymosan particles, in a concentration dependent manner, with

more efficiency compare to blank-microcapsules. Moreover, these effects were similar to those of soluble active α2m (1nM and 10nM).

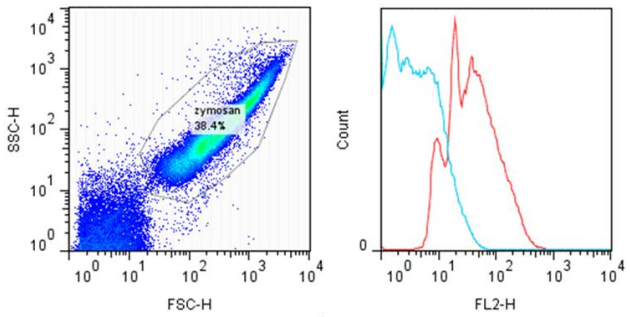


Fig. 5A. ***FACS analysis of conjugated Zymosan particles***. The particles of Zymosan were conjugated with a fluorescent dye (Bodipy, 1μM) and then verified by FACS (blue line: PBS; red line: conjugated Zymosan).

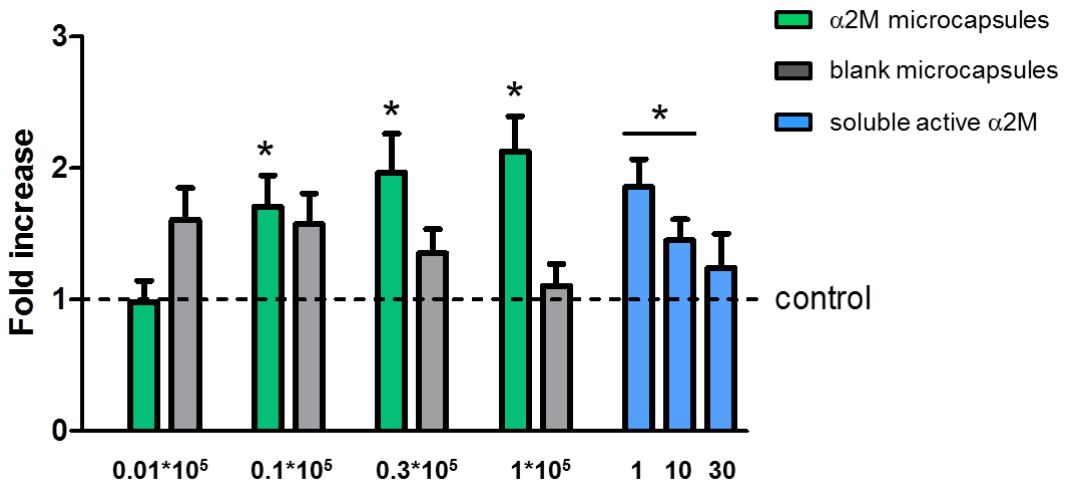


Fig 5B. ***Stimulation of MDM with α2M-microcapsules increases their ability to phagocyte Zymosan particles***. MDM were incubated with α2M-microcapsules, blank microcapsules or soluble active α2M for 24h (at 37°). The particles of Zymosan were added to cells at a final concentration of 125 µg/ml, for further 20 min. The number of phagocytosed particles was determined with a fluorescence plate reader. Data (fold increase over basal) are mean ± SEM of 3 different experiments (Unpaired t-test: *p<0.05 *vs*. control).

In another set of experiments, we studied the ability of MDM to engulf *E. Coli* particles, being this more correlated to a bacterial infection, as occur in sepsis. Therefore, MDM were first incubated with microcapsules and soluble active α2m for 24h and then fluorescent conjugated *E.Coli* particles (**Fig. 6A**) were added to cells and phagocytosis was monitored. As depicted in **Fig.**

6B, again, incubation of MDM with α2M-microcapsules significantly enhanced their ability to phagocytes bacterial particles, with more efficiency compare to blank-microcapsules, even if they seem to have some unspecific effects when used at the amount of $0.1x10^5$. Similarly to Zymosan phagocytosis, the effects of α2M-microcapsules were comparable to those of soluble active α2m (10 nM) also on *E. Coli* engulfment.

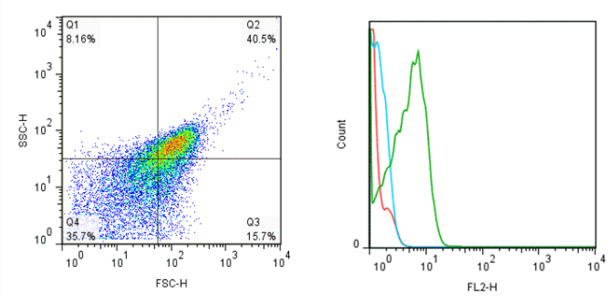


Fig. 6A. ***FACS analysis of conjugated E. Coli particles***. The particles of *E. Coli* were conjugated with a fluorescent dye (Bodipy, 1μM) and then verified by FACS (blue line: PBS; red line: unconjugated E. Coli; green line conjugated E. Coli).

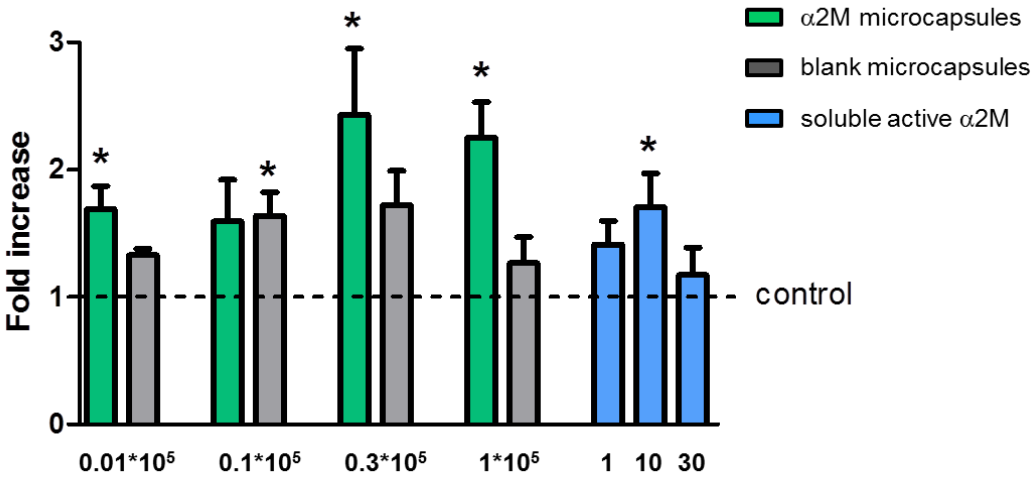


Fig 6B. ***Stimulation of MDM with α2M-microcapsules increases their ability to phagocyte E. Coli particles***. MDM were incubated with α2M-microcapsules, blank microcapsules or soluble active α2M for 24h (at 37°). The particles of *E. Coli* were added to cells at a final concentration of 1mg/ml, for further 60 min. The number of phagocytosed particles was determined with a fluorescence plate reader. Data (fold increase over basal) are mean ± SEM of 3 different experiments (Unpaired t-test: *p<0.05 *vs*. control).

DISCUSSION

The results obtained during the third year of my PhD program demonstrate that the new microcapsules enriched with active α2M display relevant biological effects. Indeed, they increase the recruitment and adhesion of neutrophils to endothelial monolayer and enhance the surface expression of α2M in these cells. Moreover, α2M-microcapsules can promote the uptake of Zymosan and E.Coli particles by human monocyte-derived macrophages.

α2M is a plasma protein with important role in the blood stream, where it can recognize and tightly bind, several growth factor and cytokines and almost all the types of proteinases. The α2M-ligand complex is cleared from circulation by binding to a classical endocytic cell-surface receptor, LRP1 or CD91. Internalized complexes may be dispatched into different pathways of endocytic/lysosomal compartment in a cell type-specific manner. The bioactive peptides bound to α2m can be degraded together with α2m or can dissociate, thereby still modulating cellular function: α2m can, therefore, modulate a variety of different biological activities and is not a merely "trapping protein". LRP1 is a member of the LDL receptor family and can bind not only activated α2m, but also several structurally and functionally unrelated ligands, being thus implicated in multiple physiological activities (Lillis *et al*., 2008).

The biological importance of α2M has further emerged after proteomic analysis of neutrophil-derived microparticles. Actually, in an unpublished work, the authors reported that α2M was abundant in a specific subset of neutrophil-derived microparticles. Moreover, this type of microparticles has been found at increased levels in the plasma of patients that survived sepsis compared to non-survivors patients or healthy donors, suggesting a key role for this protein. They demonstrated, as well, that α2M has powerful stimulatory effects on neutrophil responses. In fact, neutrophil derived microparticles containing α2M augment the adhesion of neutrophils to endothelial monolayer. This mechanism is thought to be important in sepsis, characterized in some cases by an immune paralysis and neutrophil dysfunctions that causes a predominance of an anti-inflammatory status (named compensatory anti-inflammatory syndrome-CARS). Therefore, to investigate whether the neutrophil microparticles driven pathway could be harnessed as potential therapeutic intervention, microparticles were enriched with active α2M to produce humanized nano-medicines and tested in a mouse model of sepsis, showing organ protective properties (Dalli *et al*., submitted).

Endogenous microparticles, however, contain a wide spectrum of protein, α2M being just one of few hundreds. This can explain, at least in part, the conflicting results present in literature

regarding the discrepancy in their biological functions, even when they are produced from the same cell type. Microparticles are now considered relevant signalling structures that participate in both local and long-range signalling and are involved in several physiological and patho-physiological processes. It is therefore supposed to exploit their features as endogenous controllers of the inflammatory process and to recapitulate their biological function in synthetic structures (Norling *et al*., 2011). Indeed, a new technology is currently available that allows the construction of microcapsules with defined size, composition and content of encapsulated materials (De Cock *et al*., 2010). Capsules are synthetized by layer-by-layer assembly of polymers; in particular, in this study, we used microcapsules made of poly- L-Arginine, dextran sulfate sodium salt and poly- L-Lysine. The idea was to use this new capsules enriched with key compounds of inflammation process, in order to mimic the effects of the natural microparticles on the process of interest and on specific target cells, in absence of all the others mediators present in endogenous microparticles. This can represent an advantageous tool to better understand the biological function of inflammatory mediators and, moreover, can have potential therapeutic/delivery application. The capsules used in the present study were enriched with active α2M. As explained above, this protein has some important biological properties, as in the case of sepsis, where it seems to improve neutrophils response that leads to a better outcome of the disease.

In these experiments I could observe that α2M-microcapsules mimicked the actions of neutrophil derived microparticles, and soluble α2M, displaying significant effects on both human neutrophils and monocyte-derived macrophages (MDM). Indeed, in the *in vitro* assay of leucocyte-endothelium interaction, incubation of endothelial cells with α2M-microcapsules augments neutrophils recruitment and adhesion, being these effects not reproduced using microcapsules devoid of the active protein. As showed by confocal microscopy analysis, this result can be, at least in part, due to the ability of α2m-microcapsules to increase the expression of α2m on the surface of endothelial cells.

In the second part of the project I moved to assess biological properties of these microcapsules on macrophages. This cell type has been often studied in the context of microparticle biology. For instance, atherosclerosis plaques contain microparticles that mainly derive from monocyte/macrophages and it has been also demonstrated that these microparticles can transfer the adhesion molecule ICAM-1 to endothelial cells, leading to increased leukocyte adhesion and transmigration (Mayr *et al*., 2009; Rautou *et al*., 2011). There are also evidences that monocyte-microparticles can contribute to the inflammation process and to the destruction of cartilage and bone in rheumatoid arthritis (RA) (Distler *et al*., 2005). In addition, monocyte-microparticles can induce the expression of PPARγ in monocytes and macrophages, thus switching the status of cell activation upon application of specific receptor ligands (Bardelli *et al*., 2012). Finally, macrophages

respond to α2M (Fadok *et al*., 2001) and express CD91 (Nilsson *et al*., 2012). It was therefore important to test the α2M-microcapsules on this cell type. According to our results, only α2M-microcapsules, but not blank-microcapsules, did increase the ability of macrophages to engulf Zymosan and E.Coli particles. Taken together, the effects of α2M-microcapsules can have a positive impact on the host response to sepsis and infections.

α2m-microcapsules have been used in *in vitro* assays for the first time in this study and we can conclude that they could represent a useful tool to harness the biological properties of active α2m. Indeed, this protein, despite its biological importance, has short systemic half-life (less than 4 min in mice) (Imber and Pizzo, 1981). Therefore, entrapping it in synthetic structures can be advantageous in order to increase its availability in the circulation and thus prolonging its beneficial effects on the target cells.

I propose that α2m-microcapsules could represent a valid tool with potential therapeutic/delivery applications but they have to be tested in *in vivo* models. In general terms, we have generated proof-of-concept data showing how the microcapsule technology can be used to exploit biological functions of bioactive proteins. Thus, it can be desired to further investigate other microcapsules enriched with relevant inflammation-modulating proteins, especially those that have been identified in human microparticles, for example Annexin A1, Lactoferrin and Cerulosplasmin.

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PUBLICATIONS

- Bardelli C, Amoruso A, **Federici Canova D**, Fresu LG, Balbo P, Neri T, Celi A, Brunelleschi S. Autocrine activation of human monocyte/macrophages by monocyte-derived microparticles and modulation by PPARγ ligands. Br J Pharmacol 165: 716-728 (2012).

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ABSTRACT

- Novel enriched microcapsules to deliver inflammation-modulating protein: the exemplar of α2-macroglobulin

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  William Harvey Day - **London** (October, 16th 2012).

CONGRESS PARTECIPATIONS

- William Harvey Day - St. Bartholomew's Hospital, London (October, 16th 2012).

SEMINARS

Dept. MEDICAL SCIENCE (Novara, Italy)

- *"Newtrends in allergy and immunology"* Dr. J.A. Bellanti (October, 10th 2011)

- *"Alpha-MSH and the melanocortin system ininflammation"* Prof. M. Perretti (December, 19th 2011)

- *"Galectins-carbohydrate binding protein:sweet or sour?"* Prof. M. Perretti (December, 20th 2011)

WILLIAM HARVEY RESEARCH INSTITUTE (London, UK)

- *"Endothelium specific insulin resistance leads to accelerated atherosclerosis: a role for reactive oxygen species"* Dr. Matthew C. Gage (February, 24th 2012)
- *"Macrophage migration in 3-dimensional environments: role of podosomes"* Prof I. Maridonneau-Parini (February, 27th 2012)
- *"Regulation of T cell immunity by CD31"* Prof F. Marelli-Berg (March, 12th 2012)
- *"Mesenchymal and haemopoietic stroma: a crucial liaison"* Prof. F. Dazzi (March, 15th 2012)
- *"Regulation of autoantibody production by auto-immune complexes on follicular dendritic cells"* Dr Mohey Eldin El Shikh (April, 23rd 2012)
- *"Novel mediators of progression and regression in diabetic kidney disease"* Prof. C. Godson (May, 25th 2012)
- *"Alarmins and the control of skin inflammation of psoriasis"* Dr R. Wolf (May, 28th 2012)
- *"Immunogenesis and pathogenesis of ANCA mediated vasculitis"* Prof P. Heeringa (June, 9th 2012)

OTHER ACTIVITIES:

- Royal Society Summer Science Exhibition 2012 - 3rd – 8th July 2012
- William Harvey Research Institute Annual Research Review - 11th July 2012
- Participation at Research In Progress (RIP) seminars – every 2 weeks