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NOVEL EXPERIMENTAL APPROACH TO MODULATE INFLAMMATION

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To my family

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PREFACE

My PhD project in Molecular Medicine was conducted in part in Italy and in part in England. The first and second year were spent at the Department of Medical Sciences, School of Medicine, University of Easter Piedmont A. Avogadro, Novara (Italy), studying the effects of NCX-6550 (a nitric oxide-conjugated statin) and monocyte-derived microparticles on human monocytes and macrophages. During the third year, I moved to London in the Centre for Biochemical Pharmacology, William Harvey Research Institute, Barts and The London School of Medicine, Queen Mary University of London, investigating the biological properties of alpha-2 macroglobulin-enriched microcapsules in *in vitro* assays.

As known, there is still a necessity for novel anti-inflammatory medications and new therapeutic targets to treat inflammatory diseases, especially the chronic ones. Indeed, the group of Prof. Perretti, where I have spent the third year of my PhD, is interested since long time in inflammation and especially in the mechanism that can resolve this process.

The aim of this PhD thesis was to evaluate novel experimental approaches to modulate inflammation; in particular I have assessed the effects of NO-conjugated statins, endogenous microparticles and synthetic microcapsules using several *in vitro* assays on human monocytes, macrophages, neutrophils and endothelial cells.

INTRODUCTION

1. Inflammation

"Inflammation is a response to injury of vascularized tissue. Its purpose is to deliver defensive materials (blood and fluid) to a site of injury. It is not a state but a process". [1]

Inflammation is the main response of immune system against infection, cancer and tissue injury. It's a primordial response that occurs immediately after trauma or infection and prevents or minimizes further damage to cells and tissue and finally enhances repair and healing, restoring damaged tissue to its normal physiological functioning. In general, an inflammatory response is initiated within minutes of sensing a threat and resolves within hours or days. By contrast, it is possible a breakdown in resolution of an otherwise beneficial immune response that leads to a persistent inflammation-driven disease state, so called chronic inflammation, that persists for weeks, months or even years, as in the case of rheumatoid arthritis, osteoarthritis, inflammatory bowel diseases, multiple sclerosis and atherosclerosis. The inflammatory process involves the major cells of the inflammatory-immune system, including neutrophils, mast cells, T-cells, B-cells, monocytes and macrophages and it is regulated in such a way as to ensure the appropriate leucocytes are recruited. Indeed, all the cells involved in inflammation are normally quiescent and become activated in the inflammatory focus where they produce and secrete inflammatory mediators. These extracellular mediators include cytokines, growth factors, eicosanoids (prostaglandins, leukotrienes, etc.), complement and peptides. These extracellular events regulate complex intracellular signalling pathways in the recipient cells that, at that point, coordinate a proper inflammatory response [2,3]. Among several cell types involved in inflammation, neutrophils and monocytes/macrophages are crucial for our purpose and this thesis. Briefly, neutrophils are one of the first-responders of inflammatory cells to leave the vasculature and migrate towards the site of inflammation, where they engulf and kill pathogens using oxidative and non-oxidative mechanisms. They also release different mediators, as cytokines, which in turn amplify inflammatory reactions by several other cell types. Monocytes, unlike neutrophils, are long lived cells and can differentiate to become tissue-resident macrophages or dendritic cells. They have a multitude of biological properties, including

phagocytosis, production of inflammatory mediators, initiation of immune response and scavenger abilities [1,4,5]. The active compounds secreted by inflammatory cells are therefore responsible for all the downstream reactionsin cells, and it is essential to control and modulate their actions in terms of therapeutic intervention to resolve inappropriate response of the organism, for instance in chronic inflammatory disease. Despite years of research and the variety of drugs currently available, there is still a need for new medications and novel anti-inflammatory targets.

On this regard, I dedicated my studies to Microparticles (MPs) and nitro-pravastatin. Indeed, in the last decades, several authors have focused their works on this new protagonist in the contest on inflammation, the microparticles, emphasizing its importance in this process. Moreover, the effort to modulate and ameliorate the physiological response of the organism has led to the development of new compounds that combine the anti-inflammatory actions of well-known drugs with beneficial effects of a key molecule, the nitric oxide.

2. Nitric oxide-conjugated pravastatin: a chemical strategy to modulate inflammation

During the first year of my PhD program, I was involved in the study of the effect of nitric oxide-conjugated pravastatin on monocytes and macrophages, in comparison to native drug pravastatin, focusing on key inflammatory pathways. The incorporation of a NO-moiety into the structure of know drugs can represent a clever chemical strategy to ameliorate the pharmacological properties of drugs in general, including increased safety and tolerability of the new compounds, which may be useful in case of prolonged treatment over time, such as for some inflammatory diseases as atherosclerosis or osteoarthritis.

2a. Nitric oxide

The free radical nitric-oxide (NO) is a key signalling molecule that regulates a wide range of physiological functions. It has a complex action which involves several systems, as the cardiovascular, nervous, immune, respiratory and gastrointestinal [6]. The action of NO is mediated, at least in part, by its binding to guanylate cyclase enzyme leading to its activation and the release of cGMP (cyclic guanosine monophosphate). NO can also mediate the nitrosilation or oxidation of several molecules, that then interact with different enzymes or transcription factors (e.g. nuclear factor- κB, NF-κB) [7,8]. NO is synthetized by several cell types, including endothelial cells and leucocytes, through the action of the NO-synthase enzymes (NOS) that convert L-ariginine into NO and L-citrulline. There are three types of NOS. Two of them are constitutively expressed (neuronal-NOS, nNOS, and endothelial-NOS, eNOS), while the other one is expressed only in activated cells (inducible-NOS, iNOS) and cause a rapid and significant increase of NO within the cells [9,10]. It is of considerable importance the fact that NO can have both beneficial and detrimental effects in the organism, depending on its concentration and localisation. Under normal conditions, physiological concentrations of NO (picograms or nanograms), maintained by constitutive NO synthases, can keep the activation of NF-κB suppressed, limiting in

this way processes as immune response and inflammation. In physiological condition, NO has anti-thrombotic effects, promoting vasodilation, and inhibiting platelets aggregation and smooth muscular cells migration and proliferation. Moreover, in the immune system, NO inhibits leucocytes adhesion and the release of inflammatory mediators. On the contrary, in pathological conditions, the presence of inflammatory mediators such as LPS, Tumor necrosis factor-α (TNF-α), Interferon-γ (IFN-γ) or Interleukin-1 (IL-1) leads to the activation of iNOS and to a rapid up-regulation of NO synthesis, which then increases vascular permeability and leucocytes infiltration to the site of inflammation [11]. However, persistent high concentrations of NO are toxic because it can react with superoxide anion (O_2^-) to produce extremely dangerous molecules as peroxynitrite ($ONOO^-$) or hydroxyl radical (•HO) [8,12,13] (**Fig. 1**).

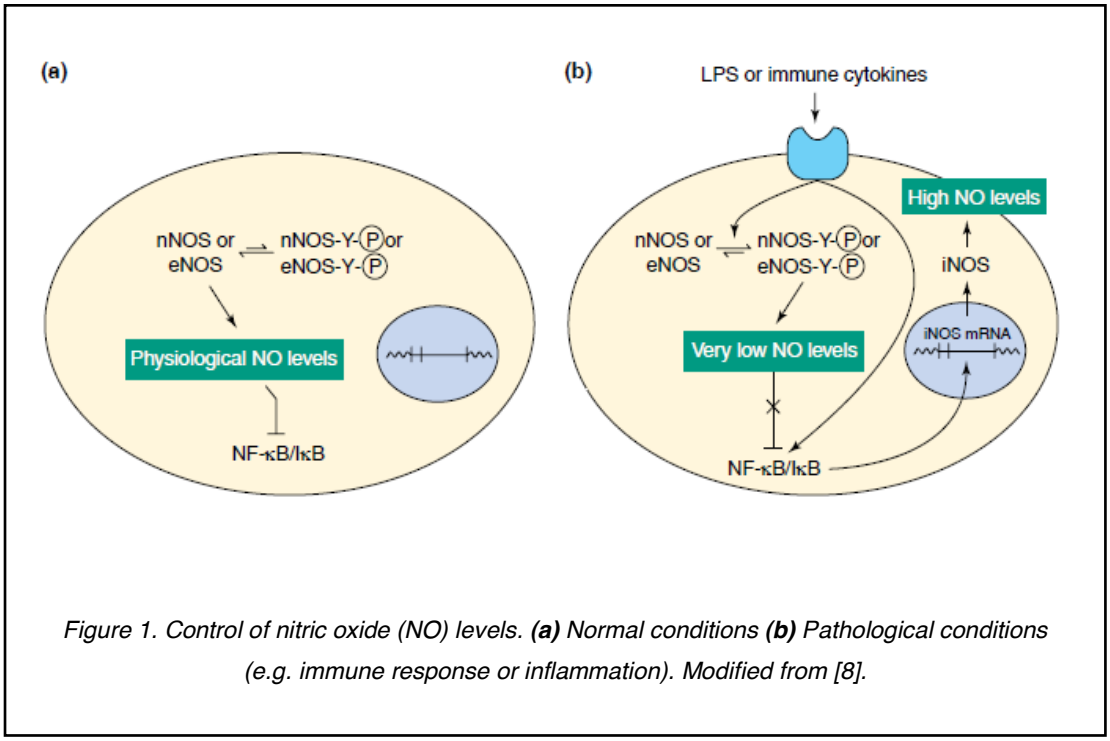


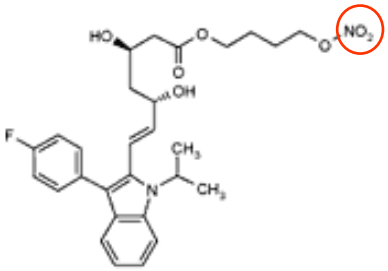
Figure 1. Control of nitric oxide (NO) levels. ***(a)*** *Normal conditions* ***(b)*** *Pathological conditions (e.g. immune response or inflammation). Modified from [8].*

2b. Nitric oxide – conjugated statins

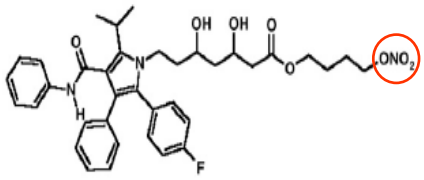
NO has been using as a therapeutic agent since many years ago, for example in the treatment of pulmonary hypertension or angina, by using nitroglycerin or isosorbide mononitrate (ISMN) [14,15], but only recently some new drugs endowed with a NO-donating moiety have been developed. The aim of this strategy is to create new compounds that can maintain the pharmacological characteristics of the native drug but can also benefit from the biological property of NO. Many studies on the biological actions of NO have focused on endothelial dysfunction since this condition plays a key role in the development of many cardiovascular diseases, including atherosclerosis, heart failure, diabetes, hypertension and hypercholesterolemia [16,17]. Regarding the last condition, closely associated with the development of atherosclerosis, inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, or statins, are the most widely used class of drugs, as they prevent the conversion of HMG-CoA to mevalonate, a precursor of cholesterol, whose high blood levels represent an important cardiovascular risk. However, it has been observed that even in individuals with normal cholesterol levels statin reduces the cardiovascular risk, suggesting the existence of mechanisms distinct from the reduction of cholesterol, the so-called pleiotropic effects of statins [18], such as anti-inflammatory, anti-proliferative and anti-thrombotic actions, resulting from the increase of NO synthesis by endothelial cells [19,20]. The anti-inflammatory role of statins has been shown in several studies using monocyte/macrophage cell lines, in which they down regulate the expression of cyclo-oxygenase-2 (COX-2) induced by LPS, the release of prostanoids and the translocation and activation of NF-κB, while increasing the activation of the nuclear receptor Perixosome Proliferation-Activated Receptor-gamma (PPAR-γ) [21,22]. Therefore, the development of compounds which combine the properties of statins with those of the NO-releasing molecules can make an additional therapeutic value, especially for those diseases (diabetes and atherosclerosis) in which a depletion of endothelial functions and consequent insufficient amounts of NO contribute to the progression of the disease.

The NicOx Research Institute Srl has long been engaged in the field of "NO-donors" molecules and has recently developed, at preclinical level, some nitro-statins. In particular, combining pravastatin and fluvastatin with NO-donating molecules, they have obtained two compounds NCX-6550 (nitro-pravastatin) and NCX-6553 (nitro-fluvastatin), which demonstrated higher lipophilicity as well as increased anti-proliferative and anti-inflammatory activity compared to native statins (**Fig. 2**).

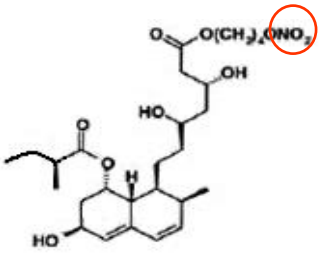
Examples of Nitro-statins



NCX-6553
(Nitro-Fluvastatin)



NCX-6560
(Nitro-Atorvastatin)



NCX-6550
(Nitro-Pravastatin)

Figure 2. Examples of NO-conjugated statins recently developed (modified from [23,24]).

Ongini's group (2004) documented that NO-statins increase, in a time-dependent manner, the concentration of nitrosyl-haemoglobin in the blood of rats; this is in agreement with a slow release kinetic of NO. NO-statins are also significantly more effective in inhibiting the proliferation of smooth muscle cells in rat arteries compared to the native compounds. Furthermore, in LPS-stimulated murine macrophage cell line (RAW 264.7), only the NO-statins can significantly decrease the expression of iNOS and COX-2, thus confirming their anti-inflammatory properties [23]. Subsequent studies have shown that they possess anti-thrombotic activity, both *in vitro* and *in vivo*, supressing platelet activation and inhibiting tissue factor expression [25]. NCX-6550 also stimulates the angiogenetic process, improving recovery after an ischemic event in type 1 diabetic mice [26]. Moreover, the nitro-pravastatin reduces the production of reactive oxygen species (ROS) in mouse models of atherosclerosis [27] and, finally,

exerts cardio-protective effects in myocardial infarction [25]. Taken together, these results indicate that the incorporation of NO moiety in the structure of pravastatin can actually exert beneficial biological effects compared to the native drug, such as improvement of endothelial function and reduction of inflammatory molecules.

In the first year of my PhD program, we studied the anti-inflammatory effects of nitro-pravastatin NCX-6550 on human monocytes and macrophages, by evaluatingits ability to interfere with inflammatory mechanisms, such as release of cytokines, superoxide anion (O_2^-) production, PPAR-γ expression and NF-κB translocation **(Paper 1)**.

3. Microparticles

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 [28] and, for a long time, they were considered residues of platelet activation or cells debris. Subsequent studies revealed that MPs 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 [29]. MPs can be generated from several cell types above platelets, including endothelial cells, monocytes, macrophages, B- and T-cells, neutrophils and erythrocytes, and present cell surface markers and cytoplasmatic components of their precursor cells [30,31]. Depending on their origin, MPs differ in size (diameter 0.1-1 μm), protein and lipid composition, and effects.

How are MPs produced? There are two well-known cellular processes that can lead to the formation of MPs: cell activation and apoptosis [29].

An increased release of MPs has been demonstrated upon stimulation of cells with different agents. Platelets, for instance, are activated by thrombin, collagen or calcium ionophore A23187 [32,33], whereas monocytes and endothelial cells release MPs after stimulation with various pro-inflammatory agents, such as bacterial lipopolysaccharide (LPS), cytokines (TNF-α or IL-1), or chemical compounds such as phorbol-myristate acetate (PMA), ionomycin or calcium ionophore A23187 [34,35]. In general, the release of MPs is time- and calcium-dependent. One of the first signs of cell activation is an increase in cytoplasmatic calcium concentration (especially on site of vesiculation), that is essential for cytoskeleton remodelling. In fact, MPs formation requires the breakdown of membrane skeleton with the consequential loss of asymmetric phospholipid distribution [36]. Apoptosis is characterized by cell contraction, DNA fragmentation and membrane blebbing and can be promoted *in vitro* by cell starvation. In apoptosis, MPs generation is associated with membrane blebbing, which depends mainly on activation of Rho-associated kinase-1, ROCK-1 [37]. ROCK-1 is a key regulator of actin organization and is also required for redistribution of fragmented DNA from the nuclear region into membrane blebs and apoptotic bodies [30].

Current wisdom is that MPs differ according to whether they are produced by either process, in term of macromolecular composition, both external and cytoplasmatic [38,39]. This suggests that MPs may not be formed through a single process, but rather that the release of MPs differs qualitatively and quantitatively, depending on parent cell types as well as on the extent of process of activation or apoptosis.

MPs are surrounded by a phospholipid bilayer that no more maintains the normal lipid asymmetry. Actually, on the external surface, MPs present negatively charged phospholipids, such as phosphatidylserine (PS) and phosphatidylethanolamine that, in resting conditions, are normally located in the inner leaflet. This exposure appears to be a nearly universal feature of cells undergoing activation or apoptosis and it likely plays a role in the *in vivo* effects of MPs, since PS can bind coagulation factors.
Concerning MPs membrane protein composition, it has been verified that MPs often carry surface antigens that are specific for the parental cell, enabling the determination of their cellular source [29,40]. For instance, CD14 is the major marker for monocyte-derived MPs, whereas CD3, CD41a and CD146 are markers for lymphocyte-, platelet- and endothelial-derived MPs respectively [31]. Beside surface components, MPs carry a broad variety of cytosolic proteins, nucleic acids and lipids that could contribute to their biological activity, although this has not been extensively investigated (**Fig. 3**).

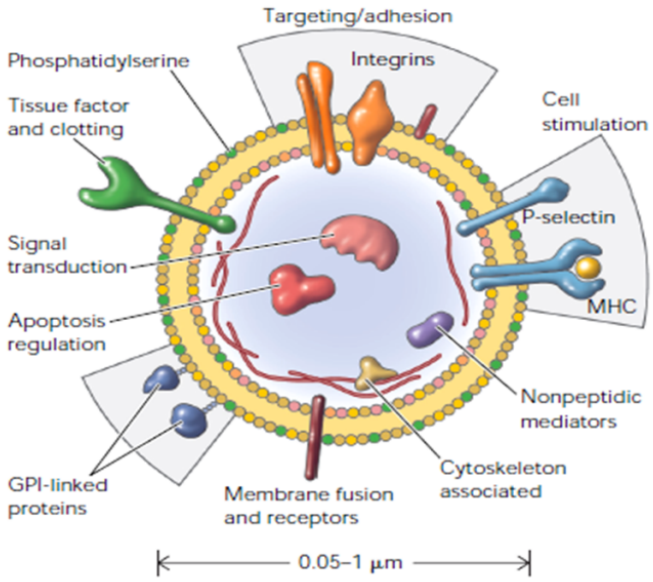


Figure 3. Schematic representation of cellular microparticles [36].

MPs are different from other subcellular structures such as apoptotic bodies and exosomes [33]. In fact, apoptotic bodies are released in the final stage of apoptosis, while MPs are generated in the early phase of the process; moreover, apoptotic bodies are larger (diameter 1 - 4 μm) compared to MPs [33,41]. On the contrary, exosomes are smaller (diameter 50-100 nm) than MPs and represent vesicles stored intracellularly in multivescicular bodies and released upon cellular activation [33,43].

The identification of MPs is, therefore, based on size and surface markers expression, flow cytometry being frequently used to analyse their features. Indeed, MPs are small structures which show characteristic forward and side scatter patterns and expose surface molecules, allowing their identification with specific antibodies by FACS. In addition, the presence of PS on the external layer of MPs membranes permits its binding with Annexin V, which is also used to identify and enumerate MPs [33].

3a. Biological importance of Microparticles in inflammation

In contrast to their initial description as cellular debris, it has become clear that MPs are novel subcellular effectors that can regulate important cellular processes, e.g. thrombosis, vascular reactivity, angiogenesis, carcinogenesis and inflammation. Indeed, MPs have been found in blood, urine, synovial fluid, extracellular spaces of solid organs, atherosclerotic plaques and tumours. MPs derived from multiple cell types can circulate in the blood and transport biologically active compounds, so participating in both local and long-range signalling, and they can also transfer and exchange surface molecules, receptors or cytoplasmatic components, thus altering signal transduction in the recipient cells (**Fig. 4**).

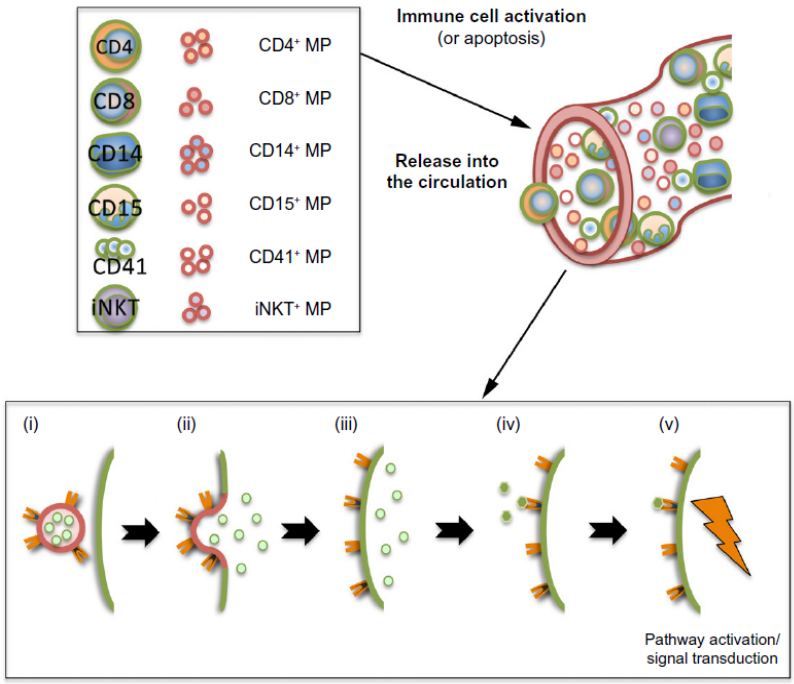


Figure 4. MPs are released from different cell types and enter in the blood stream. MPs can then fuse with plasma membrane of other cells, transferring their cargo of both cytosolic and surface molecules. Modified from [43].

In the blood of healthy donors, MPs are present at low levels and about 80% derive from platelets, so explaining why most of the studies so far performed deals with platelet-derived MPs. The level of circulating MPs is raised in a number of pathological states associated with inflammation, activated coagulation and fibrinolysis. Elevated numbers of MPs have been actually reported in several diseases, including sepsis, acute coronary syndrome, cancer, diabetes, atherosclerosis and arthritis [44]. *In vitro* studies have demonstrated that circulating MPs promote coagulation: phosphatidylserine (PS) and tissue factor (TF) are both exposed on MPs external layer, playing central role in the coagulation cascade [45]. As hypercoagulation is one of the characteristics of cardiovascular diseases, and altered numbers and pro-coagulant behaviour of MPs were reported in these conditions, MPs can play a causal role in the development of hypercoagulation in cardiovascular diseases. MPs have an impact also in vascular function. Platelet-derived MPs can transfer arachidonic acid to endothelial cells and promote COX-2 expression in these cells, thereby inducing vasodilatation, *via* an increased production of prostaglandins [32,46]. Moreover, lymphocyte-derived MPs induce endothelial dysfunction by alteration of NO and prostacyclin pathways [47]. MPs are shed even from tumour cells and they can stimulate metastasis by promoting *in vivo* angiogenesis due to the presence of sphingomyelin and TF at their external surface [48].

The implication of MPs in inflammation is well documented too. Recent studies on inflammation, have focused on MPs and reported different, and in some cases conflicting, results. In fact, in relation to the type of generating stimulus, MPs can exert pro- or anti-inflammatory effects [48,49]. Platelet-derived MPs can induce adhesion of monocytes to endothelium, since they deliver arachidonic acid to endothelial cells, which results in up-regulation of intracellular adhesion molecule-1 (ICAM-1) and prostaglandins production, *via* up-regulation of COX-2. Thereafter, monocytes migrate into the intima, where they secrete cytokines (e.g. IL-1β, TNF-α) and growth factors that promote migration and proliferation of vascular smooth muscle cells [46]. Furthermore, platelet-MPs can induce leucocyte-leucocyte aggregation, due to the interaction between P-selectin, expressed on platelet MPs, and its ligand on leucocytes [50]. In addition to platelet-derived MPs, MPs released from other cell types can exert pro-inflammatory activities. For instance, neutrophil-derived MPs induce the expression of IL-6 and monocytes chemotactic protein-1 (MCP-1) in endothelial cells (**Fig. 5**).

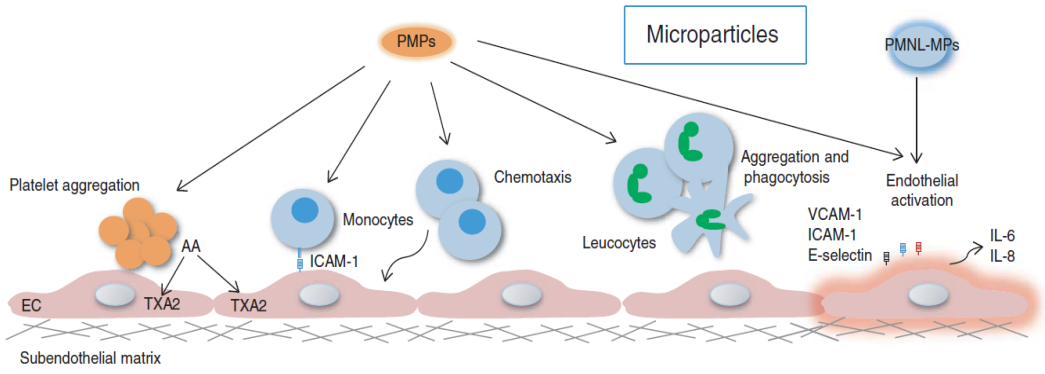


Figure 5. Pro-inflammatory potential of MPs. Platelet-derived MPs (PMPs), Polymorphonuclear leucocytes-derived MPs (PMNL-MPs) [59].

In general, MPs derived from different cell types may also play anti-inflammatory activity. For example, neutrophil-derived MPs exert anti-inflammatory effects, including inhibition of the release of IL-8 and TNF-α in macrophages, while enhancing the production of Transforming Growth Factor-β1 (TGF-β1), a potent inhibitor of macrophages activation [60]. Additionally, it has been demonstrated that neutrophils-MPs contains the functionally active anti-inflammatory protein annexin-A1, which leads to the inhibition of the interaction between leucocytes and endothelial cells both *in vitro* and in an animal model *in vivo* [31]. Paradoxically, a beneficial effect conveyed by MPs in patients with septic shock was more recently suggested by the observation that higher mortality rates and organ dysfunction were associated with lower levels of endothelial, platelet, and leucocyte-derived MPs [61]. Because MPs from patients with septic shock enhance contraction of aorta in LPS-treated mice and possibly through the delivery of thromboxane A2, an interesting hypothesis would be that MPs protect septic patients from vascular hyporeactivity by maintaining a tonic pressure response [62]. Unpublished work in Prof. Perretti's lab suggests a possible explanation for this dichotomy, between pro- and anti- inflammatory effects of MPs.Neutrophil-derived MPs 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 MPs even when produced from the same cell type.

Monocyte-derived MPs

Monocytes can release MPs upon activation with calcium ionophore or other chemical or pro-inflammatory compounds. However, monocyte-derived MPs are poorly investigated, but it has been demonstrated that they are highly pro-coagulant, primarily due to the presence of TF and PS [34,36], playing an important role in haemostasis and thrombosis. Moreover, monocytes-MPs directly activate platelets as a result of the interaction between P-selectin glycoprotein ligand-1 (PSGL-1) on MPs and P-selectin on platelets [39]. A recent study showed that the exposure of THP-1 cells (a monocytic cell line) and primary human macrophages to tobacco smoke extracts provokes the generation of highly pro-coagulant MPs that can contribute to the pathological hypercoagulability of smokers [51]. Atherosclerosis plaques contain MPs that mainly derive from monocyte/macrophages and it has been also demonstrated that these MPs can transfer the adhesion molecule ICAM-1 to endothelial cells, leading to increased leucocytes adhesion and transmigration [52,53]. There are also evidences that monocyte-MPs can contribute to the inflammation process and to the destruction of cartilage and bone in rheumatoid arthritis (RA). Indeed, monocyte-MPs induce the synthesis of matrix-metallo proteinases (MMPs) and cytokines (IL-6, IL-8) in fibroblasts isolated from patients with RA, *via* NF-κB pathway [54]. Elevated number of monocyte-derived MPs has been found in plasma of patients with lung cancer, in comparison to healthy controls, and they have been suggested to be a sign of vascular complication in these patients [55]. Moreover, it has been showed that monocyte-derived MPs affect human bronchial epithelial and alveolar cell lines [56,57]. In fact, MPs derived from human monocytes have the potential to sustain the innate immunity response of the airway epithelium, by up-regulating the synthesis of IL-8, MCP-1 and ICAM-1 in bronchial and alveolar cell lines [56]. In addition, Neri and co-authors (2011) demonstrated, in the same cell lines, that monocyte-MPs induced pro-inflammatory response that was mediated by NF-κB activation and modulated by PPAR-γ [57]. PPAR-γ is expressed by several cell types (including adipocytes, endothelial cells, monocytes/macrophages, T- and B-cells and dendritic cells) and besides representing a key transcription factor of adipocyte differentiation, lipid and glucose homeostasis, it plays an importantrole in the regulation of inflammation. Indeed, activation of PPAR-γ by selective agonists (e.g. 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2- 15d-PGJ - and rosiglitazone) induces anti-inflammatory effects, at least in part through suppression of NF-κB pathways, resulting in down-regulation of some inflammatory molecules, such as TNF-α, IL-6 and IL-1β [58].

During the second year of my PhD program I was involved in assessing the effects of monocyte-derived MPs on human monocytes and monocyte-derived macrophages (MDM) by evaluating the ability of MPs to trigger some keys inflammatory process, as oxy-radical production, cytokine release, NF-κB activation and PPAR-γ protein expression **(Paper 2)**.

3b. From Microparticles towards Microcapsules

The multiple roles of MPs and their functional importance during inflammation are 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 MPs with well-defined cargo. Several studies in the 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. These new techniques represent a drug-delivery system to carry precise quantities of a therapeutic payload at a specific target site or tissue. The method is based on layer-by-layer assembly of opposite charged macromolecules on colloidal particles to make micro- and nano-capsules with defined size, composition and content of encapsulated materials [63]. The initials steps involve stepwise formation of the film by repeated exposure of the colloids to polymers with alternating interaction. The excess polymer is removed by cycles of centrifugation and washing before the next layer is deposited. After the desired number of polymer layers is deposited, the coated particles are exposed to conditions which cause the core template to dissolve (**Fig. 6**). 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 refilled with substances of interest [64,65]. 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. These layers are deposited around a core of $CaCO_3$ that, at the end of the process, is dissolved using ethylenediaminetetra-acetic acid (EDTA) [65,66]. 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 [67]. The external shell can also incorporate a fluorescent probe, making the microcapsules visible under a fluorescent microscope.

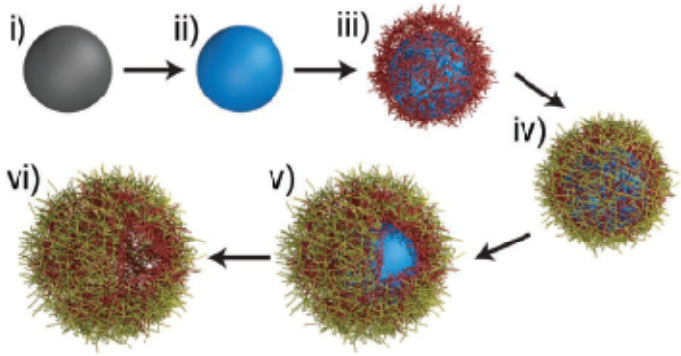


Figure 6.Schematic representation of layer-by-layer assembly of microcapsules [63].

One of the mayor promising fields of application of this technique can be related to delivering proteins that can modulate inflammatory process. Proteins are commonly administrated by injection, as other routes often suffer from poor bioavailability. Most proteins, however, have limited stability or short half-life when applied *in vivo*, requiring, in this case, multiple administrations. Encapsulation of proteins can therefore enhance their stability and targeting towards the site of action. Obviously, this process should not affect the biological activity of the encapsulated mediators.

Thus, this capsules technology' can allow mimicking the endogenous MPs, and 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.

3c. α2M, LRP-1 and α2M-microcapsules

Among the several mediators of the inflammatory process discovered in MPs, 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 represents 8-10% of total serum protein, and its level in the blood stream is around 2-4 mg/ml. Several cell types can synthetize α2M, as hepatocytes, fibroblasts, monocytes, macrophages and astrocytes, and TGF-β and IL-6 are reported to increase its levels [68]. 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 [68-70]. α2M-carrier interactions are mainly reversible in nature and, as a result, α2M may inhibit growth factor activity [71,72] or stabilize the growth factor for possible delivery to cell signalling receptors [73]. The native or inactive 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 (**Fig. 7**), and the entire complex binds to the α2M receptors, nowadays termed low density lipoprotein receptor like protein-1 (LRP-1; [74]). The protease–α2M-receptor complex is then internalized and can undergo lysosomal degradation, with the receptor being eventually recycled to the cell surface [68].

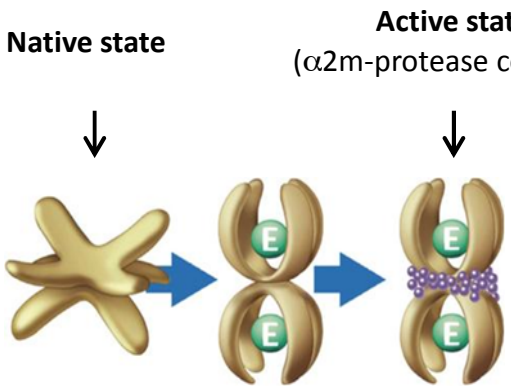


Figure 7. Schematic representation of α2m in its native state and after entrapping an enzyme (E).

Binding of α2M to LRP-1 can activate ERK/MAP kinase and Akt and thereby promotes cell survival and migration, for instance in Schwann cell [75]. LRP-1 is a member of LDL receptor gene family and it is a ubiquitous type 1 transmembrane receptor, whose systemic expression is essential for embryonic development and for maintenance of basal cellular function and development and survival of the organism. LRP-1 can recognize at least 40 different ligands, such as apolipoprotein E (apoE) containing lipoproteins, lipoprotein lipase, plasma proteins (e.g. urokinase-type tissue plasminogen activator), hormones, matrix proteins, and growth factors like PDGF; it is endowed with scavenger properties and it now seems, can also signal, through its cytoplasmatic tail that can bind multiple intracellular adapter and scaffold proteins. It is no surprise then that this receptor is being implicated in numerous processes (e.g. lipid metabolism, proteinase metabolism, activation of lysosomal enzymes and neurotransmission). Several studies dealt with its involvement in the modulation of the progression of Alzheimer's disease by binding β-amyloid precursor protein (APP), whose deposition in the brain causes several neuronal dysfunctions. Indeed, LRP-1 mediates outward transport of APP from the brain across the blood-brain barrier (BBB) and plays a key role in clearing APP to the blood circulation [76-80]. Moreover, in several studies, the receptor has been associated with a clear role in the pathogenesis of atherosclerosis. In the liver it has been shown that the receptor is important for the removal of atherogenic lipoproteins and other pro-atherogenic ligands from the circulation [81,82]. The mechanism by which LRP-1 protects against the formation of atherosclerotic lesions is mediated through the control of at least two distinct signalling

pathways in vascular smooth muscle cells (vSMCs): the platelet-derived growth factor (PDGF) and the TGF-β signalling pathways, which both play major roles during atherosclerosis [83,84]. Macrophages express LRP-1 and play a central role in the pathogenesis of atherosclerosis by internalizing modified low-density lipoprotein (LDL), producing cytokines and growth factors, stimulating therefore the migration and proliferation of smooth muscle cells and plaque development and progression [85]. Some authors showed that LRP-1 deficiency leads to a significant increase in atherosclerosis, being macrophages involved through the modulation of the extracellular matrix and inflammatory responses [86,87]. LRP-1 has been also reported to mediate macrophages phagocytosis of apoptotic cells, through the interaction between calreticulin on the apoptotic cells and the receptor on the engulfing cells [88]. Moreover, a class of anti-inflammatory drugs, glucocorticoids, can up-regulate LRP-1 expression in macrophages and therefore enhance their ability to uptake apoptotic cells [89].

α2M–LRP-1 axis has also 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, involved in the pathogenesis of several diseases, is thought to be essential in certain conditions, including sepsis [70,90].

In unpublished work, that forms the foundation of the project of the third year of my PhD program (α2m-microcapsules section), α2M has been found to be abundant in a specific subset of neutrophils derived MPs, and to be a major determinant for their protective effects in sepsis. The presence of this plasma protein in MPs could be a consequence of its uptake through pinocytosis by neutrophils and incorporation into secretory vesicles [91]. Sepsis is characterized by an immune paralysis phase, with neutrophils dysfunction, leading to predominance of an anti-inflammatory status (compensatory anti-inflammatory syndrome-CARS) [92]. Neutrophil-derived MPs positive for α2M were significantly abundant in the plasma of sepsis-survivor patients compared to non-survivor and healthy volunteers. Additionally, these MPs enriched with α2M acted as nano-medicines with potent organ protective properties in a model of murine sepsis, where they reduced bacterial load in peritoneal exudate and blood, decreased neutrophils 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, Dalli *et al*. demonstrated that exposure of endothelium to α2M-enriched MPs can increase the extent of neutrophil adhesion.

Since α2M is a determinant of MPs and plays a crucial role in severe diseases, including sepsis, this project aimed at exploiting the capsules technology to recapitulate the biological function of this protein in synthetic structures. This can help to mimic the endogenous MPs 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 MPs. Furthermore, this could represent an important step in manufacturing microcapsules enriched with biologically relevant proteins, hence with potential therapeutic/delivery application (α2M-microcapsules section).

MATERIALS and METHODS

The methods used in the two published papers can be seen in the specific "Material and Method" section of the papers. In this paragraph, I detail the methods used in the unpublished work on α2M-microcapsules.

Generation and characterization of α2M enriched-microcapsules

α2M enriched-microcapsules were generated by the group of Sukhorukov (SEMS) along their established procedures [65]. 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. Platelet-rich plasma (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 gcm^{-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 [58,93]. 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℃, 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 [94-96].

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 [97] and collected in T75 flasks in complete medium (M199) supplemented with penicillin (100U), streptomycin (100mg/ml), amphotericin B (2.5 μg/ml), L-glutamine (2mM) and 20% of

human serum (HS, Lonza). HUVEC were used at passage 1 or 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℃. 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 (**Fig. 8**). The total number of interacting neutrophils was quantified as captured and further classified as rolling or adherent if stationary for the 10 sec period [98].

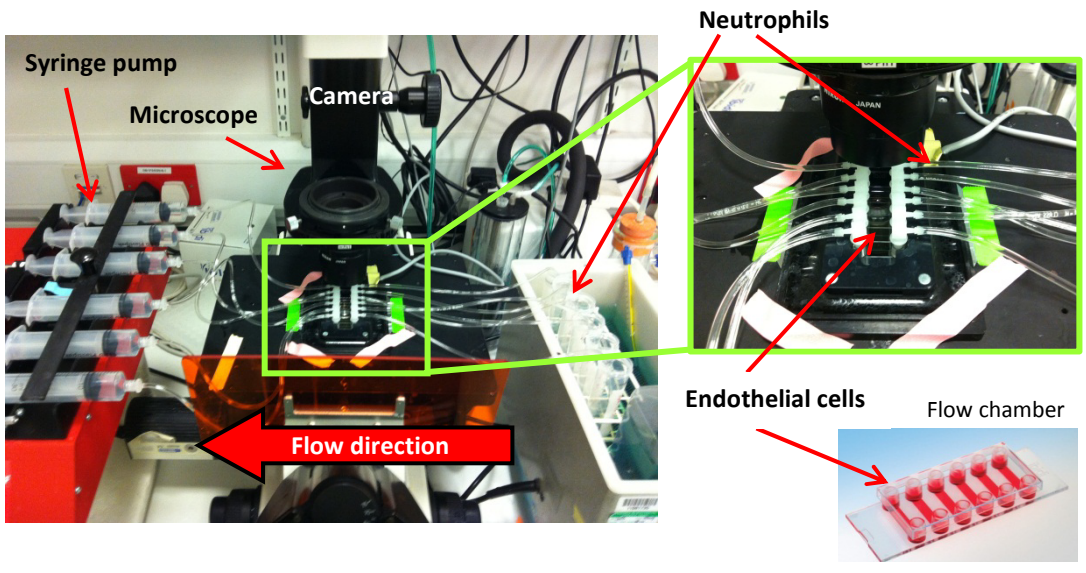


Figure 8. Flow chamber system. Neutrophils are resuspended and perfused over the endothelial monolayer and then collect through a syringe pump system.

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, AbDSerotec) 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 FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA) using CellQuest TM 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 *µ*g/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

PAPER (1)

"The nitric oxide-donating pravastatin, NCX 6550, inhibits cytokine release and NF-κB activation while enhancing PPAR-γ expression in human monocyte/macrophages"

Amoruso A, Bardelli C, Fresu LG, Poletti E, Palma A, Federici Canova D, Zeng HW, Ongini E, Brunelleschi S.

Pharmacol Res. 2010; 62: 391-9

New pharmacological agents that combine the properties of inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, statins, with those of a nitric oxide (NO)-releasing compound, have been recently developed. The new nitro-statin might provide added therapeutic value, especially in those pathologies such as diabetes and atherosclerosis, where an impairment of endothelial function causes the inability to produce endogenous NO and plays a crucial role in disease progression. Previous studies have shown that NCX-6550, a NO-donating pravastatin, induces potent anti-inflammatory effects in murine macrophage cell lines [23]. Notably, this nitro-statin maintains HGM-CoA reductase inhibition properties, still preventing cholesterol synthesis, as showed in a previous work [99]. Hence, in this study we aimed to assess its anti-inflammatory potential in human monocytes and macrophages from healthy donors, in comparison to the native drug pravastatin. We first reported that NCX-6550 inhibits, in a concentration-dependent manner, superoxide anion production in PMA-stimulated monocytes. We also showed its ability to down regulate, in a concentration dependent manner, PMA-induced TNF-α and IL-6 release, which represent major biomarkers of cardiovascular risk. Among the different signal transduction pathways involved in cytokine secretion, we focused our attention on NF-κB and PPAR-γ, functionally active in human monocyte/macrophages [94,96,100]. According to our results, NCX-6550 inhibits, in a concentration-dependent fashion, PMA-induced NF-κB activation and transmigration to the nucleus. These effects were reproduced also with PPAR-γ, agonist, 15d-PGJ. NCX-6650 also significantly enhances PPAR-γ mRNA

levels and protein expression in monocytes, with similar effects as 15d-PGJ. In all the experiments, pravastatin was significantly less efficient compared to nitro-statin. In conclusion, the major ability of NCX-6550 to induce PPAR-γ expression, as well as its capability to inhibit PMA-induced cytokine release, oxy-radical production and NF-κB translocation, compared to the native pravastatin, clearly indicate that this NO-donating statin might have relevant beneficial effects for the treatment of cardiovascular inflammatory disorders, such as atherosclerosis.

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The nitric oxide-donating pravastatin, NCX 6550, inhibits cytokine release and NF-κB activation while enhancing PPARγ expression in human monocyte/macrophages

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ABSTRACT

Previous studies have shown that NCX 6550 (NCX), a nitric oxide (NO)-donating pravastatin, induces anti-inflammatory effects in murine macrophage cell lines. Here, we have studied its activity in human monocyte/macrophages, by investigating cytokine release, NF-κB translocation and peroxisome proliferator-activated receptor γ (PPARγ) expression and function. For comparison, pravastatin, isosorbide-5-mononitrate (ISMN), sodium nitroprusside (SNP) and the PPARγ ligand 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (PGJ) were also tested.

Monocytes and macrophages (MDM: monocyte-derived macrophages) were isolated from healthy donors; cytokine release was measured by ELISA, NF-κB by electrophoretic mobility shift assay and PPARγ by Western blot and Real-Time PCR.

NCX (1 nM–50 μM) dose-dependently inhibited phorbol 12-myristate 13-acetate (PMA)-induced TNF-α release from monocytes (IC_{50} = 240 nM) and MDM (IC_{50} = 52 nM). At 50 μM, it was more effective than pravastatin, ISMN and SNP ($P < 0.05$), but less efficient than PGJ. Similar results were obtained for IL-6. Likewise, NCX was more effective than pravastatin and the other NO donors in inhibiting PMA-induced NF-κB translocation in both cell types, and, at the highest concentration, significantly ($P < 0.05$) enhanced PPARγ protein expression in monocytes.

We conclude that NCX 6550 exerts a significant anti-inflammatory activity in human monocyte/macrophages, that is also contributed by its NO donating properties, as the effects exerted by NCX are significantly higher than those evoked by pravastatin in many experimental assays. These data further indicate that the incorporation of a NO-donating moiety into a statin structure confers pharmacological properties which may translate into useful therapeutic benefits.

1. Introduction

There is evidence that grafting a nitric oxide (NO)-releasing moiety to well established drugs enhances their anti-inflammatory potential and confers additional properties, in a variety of models [1–4]. Human monocyte/macrophages play a key role in inflammation and chronic diseases and represent an interesting model to evaluate the effects of NO donating drugs. In fact, although human cells express the iNOS (inducible NO synthase) gene, their NO production differs markedly from that shown in murine macrophages or macrophage cell lines [5,6]. While treatment of rodent macrophages with IFN-γ, TNF-α or lipopolysaccharide (LPS) results in a rapid and abundant production of NO, the same stimuli fail to induce the appearance and accumulation of NO degradation products in cultured human monocyte/macrophages [5,6].

Abbreviations: EMSA, electrophoretic mobility shift assay; IL-6, interleukin-6; ISMN, isosorbide-5-mononitrate; MDM, monocyte-derived macrophage; NCX, NCX 6550: 1S-[1α(βS*,δS*),2α,6α,8β-(R*),8aα]-1,2,6,7,8,8a-hexahydro-β,δ,6-trihydroxy-2-methyl-8-(2-methyl-l-oxobutoxy)-1-naphtalene-heptanoic acid 4-(nitrooxy)butyl ester; NF-κB, nuclear factor-kappa B; NO, nitric oxide; PGJ, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2; PMA, phorbol 12-myristate 13-acetate; PPARγ, peroxisome proliferator-activated receptor-gamma; SNP, sodium nitroprusside; TNF-α, tumour necrosis factor-alpha.

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Previous work on the NO-donating pravastatin NCX 6550 showed that the drug exerts potent anti-inflammatory activities in RAW 264.7 cells, a murine macrophage cell line [4], has anti-thrombotic activity and inhibits tissue factor expression, so reducing the generation of pro-coagulant activity by murine peritoneal macrophages [7]. It has also been reported that NCX 6550 stimulates angiogenesis and improves recovery from limb ischaemia in diabetic mice [8], reduces the generation of reactive oxygen species in normal and atherosclerotic mice and demonstrates a superior anti-inflammatory effect compared to pravastatin [9,10]. With this background, it was critical to understand whether such results could be validated in human tissues.

There is evidence that, besides lipid lowering, statins exert beneficial effects through cholesterol-independent properties, including anti-inflammatory activity [11–13]. For example, in monocyte/macrophage cell lines, statins have been shown to inhibit LPS-induced cyclooxygenase-2 (COX-2) expression, prostanoid release and Nuclear Factor-kappa B (NF-κB) translocation [14], and to increase peroxisome proliferator-activated receptor (PPAR)-γ activity [15].

The role of PPARγ in regulating inflammatory processes and atherosclerosis is widely accepted. The anti-inflammatory potential of PPARγ agonists mainly resides in their ability to inhibit monocyte/macrophage activation and the expression of inflammatory molecules, such as TNF-α, IL-6, IL-1β, iNOS, gelatinase B and COX-2 [16–18]. Therefore, PPARγ has been suggested to mediate some of the pleiotropic actions of statins [19].

Hence, we have assessed the anti-inflammatory potential of NCX 6550 and compared it with that elicited by the reference pravastatin in monocytes and macrophages (MDM: monocyte-derived macrophages) from healthy donors. We investigated the ability of NCX 6550 to modulate cytokine release, NF-κB nuclear translocation and PPARγ expression, as compared to pravastatin, sodium nitroprusside (SNP; a direct NO donor [2]), isosorbide-5-mononitrate (ISMN; a NO donor requiring metabolism [2]), and the PPARγ ligand 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (PGJ).

Here we demonstrate that NCX 6550 enhances PPARγ protein expression and inhibits NF-κB activation and cytokine release induced by the protein kinase C (PKC) activator, phorbol 12-myristate 13-acetate (PMA). These effects are dose-dependent and, at the maximal 50 μM concentration, NCX 6550 is significantly more effective than pravastatin, SNP and ISMN.

2. Materials and methods

2.1. Cell culture

Human monocytes were isolated from heparinized venous blood (30–40 ml) of healthy donors by standard techniques of dextran sedimentation, Hystopaque (density = 1.077 g/cm^3) gradient centrifugation (400 × *g*, 30 min, room temperature) and recovered by thin suction at the interface, as described elsewhere [16]. Cells were resuspended in RPMI 1640 medium, supplemented with 5% heat-inactivated foetal bovine serum (FBS), 2 mM glutamine, 50 μg/ml streptomycin, 5 U/ml penicillin and 2.5 μg/ml amphotericin B; purified monocyte populations were obtained by adhesion (90 min, 37 °C, 5% CO_2). Cell viability (trypan blue dye exclusion) was usually > 98% and was not affected by the different compounds under evaluation (data not shown).

Monocyte-derived macrophages (MDM) were prepared from monocytes, by culture (8–10 days) in RPMI 1640 medium containing 20% FBS, glutamine and antibiotics; medium was exchanged every 2–3 days [16]. MDM were defined as macrophage-like cells by evaluating surface markers CD14, MHCII, CD1a and CD68 [20]. Briefly, adherent cells were detached by gentle scraping. After three washings with sterile phosphate-buffered saline (PBS), cells were resuspended at the final concentration of 1×10^5 cells/ml and fluorescent dye-labelled antibodies against the different surface markers (anti-CD14 from Becton Dickinson, Oxford, UK; anti-CD68 and anti-MHCII from Dako, Milan, Italy; anti-CD1a from Bioscence, San Diego, CA, USA) were added for 30 min on ice and the reaction was stopped with 4% paraformaldehyde, according to the manufacturer's instructions. Incubation was performed in the dark and expression of surface markers was analysed by flow cytometry.

A different number of cells was used according to the type of experiments (2×10^6 cells for Western blot experiments, 5×10^6 cells for EMSA assays and 1×10^6 cells for cytokine release). The murine macrophage RAW 264.7 cells (ATCC, Manassas, VA, USA), which were used for comparison in some experiments (see below), were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS, glutamine and antibiotics.

To rule out toxic effects of the different drugs, we evaluated the cell viability by the methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay, in addition to the trypan blue dye exclusion evaluations. In these experiments, monocytes (1×10^5) were challenged with the maximal concentration of the compounds for 6 or 24 h. Thereafter, the medium was replaced by the MTT solution (1 mg/ml) after 2 h incubation at 37 °C in the dark. The supernatant was removed and DMSO was added in order to dissolve the purple formazan; the absorbance of the samples was read at 580 and 675 nm. All the compounds did not reduce *per se* monocyte viability, absorbance values being always around 0.11 (MTT assay), as control cells.

2.2. Nitrite accumulation and superoxide anion (O_2^-) production

We first characterized the effects of NCX 6550 on the NO pathway and oxy-radical production. Since human monocytes are known to release very low amounts of NO [5,6], the murine macrophage cell line RAW 264.7 was used as a positive internal control. Nitrite content was determined in 100 μl of cell culture medium by the Griess colorimetric reaction, using "Griess Reagent System", according to manufacturer's instructions (Promega, Madison, USA).

Briefly, 1×10^5 RAW 264.7 cells and 1×10^5 human monocytes were pre-treated for 30 min with NCX 6550 at the maximal 50 μM concentration (see later) and then challenged with phorbol 12-myristate 13-acetate (PMA) or lipopolysaccharide (LPS) for 30 min or 24 h. PMA was used at 10^{-6} M (30 min) or 10^{-7} M (24 h); LPS was used at 100 ng/ml (30 min) or 10 ng/ml (24 h). These two time-points were selected according to the optimal times required to induce superoxide anion production (30 min) and cytokine release (24 h) in human monocyte/macrophages.

To evaluate superoxide anion (O_2^-) production, human monocytes and RAW 264.7 cells (1×10^6 cells/plate) were washed twice with PBS, and stimulated, in the absence or presence of NCX 6550, by PMA 10^{-6} M or LPS 100 ng/ml for 30 min. O_2^- production was evaluated by the superoxide dismutase (SOD)-inhibitable cytochrome *C* reduction, the absorbance changes being recorded at 550 nm in a Beckman DU 650 spectrophotometer. O_2^- production was expressed as nmol cytochrome *C* reduced/10^6 cells/30 min, using an extinction coefficient of 21.1 mM [21]. To avoid interference with spectrophotometrical recordings of O_2^- production, cells were incubated with RPMI 1640 without phenol red, antibiotics and FBS.

2.3. PPARγ protein expression in monocyte/macrophages and semi-quantitative analysis

PPARγ expression in human monocytes and MDM was evaluated by Western blot, as described [16,17,20]. Accordingly, cells

were evaluated either as they stand (i.e., "basal, constitutive PPARγ expression") or after challenge (6 h, 37 °C, 5% CO_2) with NCX 6550, pravastatin, sodium nitroprusside (SNP), isosorbide-5-mononitrate (ISMN) or the endogenous PPARγ ligand PGJ, for comparison. The 6 h challenge period was found to be the optimal one [20]. Cells were washed twice with ice-cold PBS and scraped off the wells in lysis buffer containing 3% SDS, 0.25 M Tris and 1 mM phenyl-methyl-sulfonyl fluoride (PMSF). Cells were lysed by sonication; when necessary, cell lysates were stored at −80 °C. The determination of protein concentration was done by the bicinchoninic acid assay (BCA). Protein samples (20 μg) were analysed by SDS-PAGE (10% acrylamide) and electro-blotted on nitrocellulose membrane (Protran, PerkinElmer Life Sciences). Immunoblots were performed according to standard methods using the following antibodies: monoclonal mouse anti-human PPARγ (E-8; Santa Cruz, USA; 1:1000 in TBS-T 5% milk) and monoclonal mouse anti-human β-actin (Sigma, USA; 1:5000 in TBS-T 3% BSA), as described [16,20]. Anti-mouse secondary antibody was coupled to horseradish peroxidase (Amersham Biosciences, UK). Proteins were visualized with an enzyme-linked chemiluminescence detection kit according to the manufacturer's (PerkinElmer) instructions. Chemiluminescence signals were analysed under non-saturating conditions with an image densitometer (Versadoc, Bio-Rad, USA). Semi-quantitative evaluation of PPARγ protein was performed as described [16,20], by calculating the ratio between its expression and the expression of the reference housekeeping protein, β-actin.

2.4. RNA isolation and Real-Time PCR

Total RNA was extracted from monocytes with the GenElute Mammalian Total RNA Miniprep kit (Sigma, USA), according to the manufacturer's instructions. The amount and purity of total RNA was spectrophotometrically quantified by measuring the optical density at 260 and 280 nm. cDNA synthesis was performed using the High Capacity cDNA Reverse Transcription kit (Applied Biosystem; Carlsbad, CA) according to the manufacturer's instructions. Monocytes were challenged with NCX 6550, pravastatin, SNP, ISMN or the selective PPARγ ligands, PGJ and rosiglitazone, for 2 h. Real-Time PCR was carried out in a volume of 20 μl per well in a 96-well Optical Reaction Plates (Applied Biosystem) containing 1 μl TaqMan Expression Assay (PPARγ), 5 μl RNase free water, 10 μl TaqMan Universal PCR MasterMix (2×) (without AmpErase UNG) and 4 μl cDNA template. The plate was run on the Applied Biosystem 7000 ABI Prism System. To compensate for variations in cDNA concentrations and PCR efficiency between tubes, an endogenous gene control (beta-glucuronidase, GUSB) was included for each sample and used for normalization [22]. Results were analysed by the comparative cycle threshold method, according to Livak and Schmittgen [22].

2.5. NF-κB activation

The evaluation of NF-κB activation was performed by electrophoretic mobility shift assays (EMSA), as described [21]. Cells were pre-treated for 30 min with NCX 6550, pravastatin, SNP, ISMN, PGJ or vehicle, and stimulated by PMA 10^{-6} M for 1 h. According to previous experience, monocyte/macrophages challenged with PMA 10^{-6} M for 1 h presented a maximal NF-κB nuclear translocation [23]. Nuclear extracts were prepared by using "Nuclear Extraction kit" (Active Motifs, Rixensart, Belgium). Briefly, cells were resuspended in hypotonic buffer, lysed with 5% (v/v) detergent and centrifuged for 30 s at 14,000 × *g* in a microcentrifuge pre-cooled at 4 °C. Supernatant (cytoplasmic fraction) was recovered in a new tube; the pellet (nuclear fraction) was resuspended in lysis buffer and incubated for 30 min on ice on a rocking platform (150 rpm). Nuclear fraction was then centrifuged (10 min, 14,000 × *g*, 4 °C) and the supernatant (nuclear extract) transferred and stored at −80 °C until use.

Nuclear extracts (5 μg) were incubated with 2 μg poly (dI-dC) and the [γ-^{32}P]ATP-labelled oligonucleotide probe (100,000–150,000 cpm; Promega) in binding buffer (50% glycerol, 10 mM Tris–HCl, pH 7.6, 500 mM KCl, 10 mM EDTA, 1 mM dithiothreitol) in a final volume of 20 μl for 30 min at room temperature. The NF-κB consensus oligonucleotide (5′-AGTTGAGGGGAC TTTCCCAGGC-3′) was obtained from Promega. The nucleotide–protein complex was separated on a 5% polyacrylamide gel in 0.5 × TBE buffer (100 mM Tris–HCl, 100 μM boric acid, 2 mM EDTA) at 150 V on ice. The gel was dried and radioactive bands were detected by autoradiography [21]. Supershift assays were performed using a commercial antibody (anti-NF-κB p65(F6): sc-8008) from Santa Cruz Biotechnology, Inc., (CA, USA).

2.6. Cytokine release

Cytokine release was measured by enzyme-linked immunoassay kit (Pelikine Compact™ human ELISA kit; CBL, Netherlands), as previously reported [16,20,21]. TNF-α and IL-6 were evaluated as the most relevant pro-inflammatory cytokines, whereas IL-10 was selected as the reference anti-inflammatory cytokine. The amount of each cytokine was expressed in pg/ml, as indicated by the manufacturer's instructions. For these experiments, cells (1×10^6) were pre-treated for 1 h with NCX 6550 (1 nM–50 μM) and then stimulated by PMA 10^{-7} M for 24 h. According to previous experiments [20,21], the 24 h challenge period ensured the maximal cytokine release. NCX 6550 effects on cytokine release were compared with those elicited by PGJ (1 nM–50 μM), and the reference substances pravastatin, SNP and ISMN, all used at 50 μM.

2.7. Materials

FBS was from Gibco (Paisley, UK). PBS, Hystopaque, RPMI 1640 medium, glutamine, Hepes, streptomycin, penicillin, amphotericin B, protease inhibitors, SOD, cytochrome *C*, MTT, Poly (dI-dC) and monoclonal mouse anti-human β-actin antibodies were obtained from Sigma (St Louis, USA). The monoclonal mouse anti-human PPARγ (E-8) antibody was from Santa Cruz (CA, USA). Rosiglitazone and PGJ were from Cayman (Ann Arbor, USA). NCX 6550, pravastatin, SNP and ISMN were kindly provided by NicOx Research Institute, Bresso, Milan (Italy). They were dissolved in DMSO and then diluted in buffer and used at a maximal 50 μM concentration, so that DMSO final concentration did not exceed 0.5% and did not interfere with the assays reported here (not shown). Tissue-culture plates were from Nunc Ltd. (Denmark); all cell culture reagents, with the exception of FBS, were endotoxin-free according to details provided by the manufacturer.

2.8. Statistical analysis

All statistical analyses were performed using SPSS statistical software (version 15.0, SPSS Inc., Chicago, IL). Data are mean ± S.E.M. of '*n*' independent experiments. Concentration-effect curves for NCX 6550 and PGJ were constructed and their IC_{50} values (on PMA-induced cytokine release) were interpolated from curves of best fit. Statistical evaluation was performed by one-way analysis of variance between groups (ANOVA) followed by Bonferroni *post hoc* test or Student's *t*-test when appropriate. Differences were considered statistically significant when $P < 0.05$.

3. Results

3.1. Effects of NCX 6550 on nitrite accumulation and oxy-radical production in human monocytes

NCX 6550 was previously demonstrated to produce, in whole rat blood experiments, a linear time-dependent increase in nitrosylhemoglobin formation, which is consistent with a slow NO release kinetics [4]. In murine macrophage RAW 264.7 cells stimulated by IFNγ and LPS, it was also shown to significantly reduce iNOS protein expression, as well as nitrite accumulation [4]. Therefore, we have evaluated whether NO release could also be evidenced in human monocytes. In these experiments, human monocytes and RAW 264.7 cells (shown for comparison) were challenged with NCX 6550 at 50 μM for 30 min, and then stimulated by PMA or LPS for further 30 min or 24 h.

As reported in Fig. 1A, human monocytes spontaneously release low amounts of nitrite (one of the inactive oxidation products derived from NO), thus confirming data from the literature [5]. It is also worth noting that human monocytes challenged with NCX 6550 for 30 min displayed a small, although significant ($P < 0.05$), increase in nitrite accumulation as compared to control monocytes (Fig. 1A). In RAW 264.7 cells, PMA or LPS did not affect nitrite accumulation after 30 min challenge, but significantly increased nitrite production after 24 h, as shown previously [4]. In keeping with previous data [4], NCX 6550 potently inhibited nitrite accumulation in activated RAW 264.7 cells challenged with stimuli for 24 h. Moreover, PMA-stimulated monocytes (but not LPS-treated cells) exhibited a significantly ($P < 0.05$ *vs* control) increased nitrite accumulation after 30 min and 24 h challenge (Fig. 1A); this suggests that, in human monocytes, PMA is able to induce *de novo* synthesis of iNOS protein and, consequently, nitrite accumulation in the cell culture medium, as evidenced in other cell types [24]. Interestingly, unlike pravastatin (used at the same 50 μM concentration; not shown), NCX 6550 inhibited nitrite accumulation ($P < 0.05$) in both RAW 264.7 cells and PMA-stimulated human monocytes (Fig. 1A).

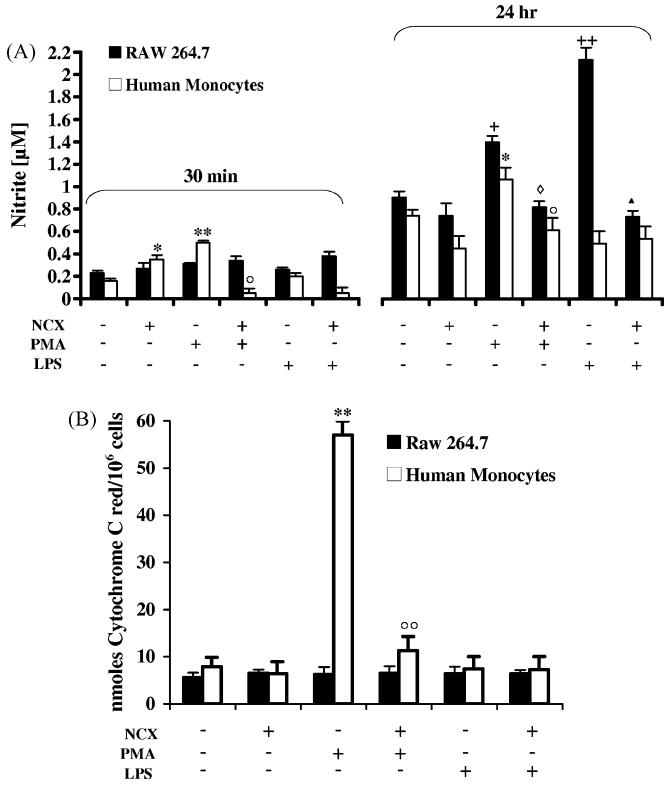

Fig. 1. Effects of NCX 6550 on nitrite accumulation (1A) and oxy-radical production (1B). In A: nitrite accumulation. RAW 264.7 cells (■) and human monocytes (□) were treated with or without NCX 6550 (NCX, 50 μM) and then stimulated by PMA or LPS for 30 min or 24 h. Values are mean ± S.E.M.; $n = 3$. $^{*}P < 0.05$ *vs* control, un-stimulated monocytes; $^{**}P < 0.01$ *vs* control, un-stimulated monocytes; $^{\circ}P < 0.05$ *vs* PMA-stimulated monocytes; $^{+}P < 0.05$ *vs* control, un-stimulated RAW 264.7 cells; $^{++}P < 0.01$ *vs* control, un-stimulated RAW 264.7 cells; $^{\diamond}P < 0.05$ *vs* PMA-stimulated RAW 264.7 cells; $^{\blacktriangle}P < 0.05$ *vs* LPS-stimulated RAW 264.7 cells. In B: superoxide anion production. RAW 264.7 cells (■) and human monocytes (□) were treated with or without NCX and then stimulated by PMA or LPS for 30 min. Values are mean ± S.E.M.; $n = 4$. $^{**}P < 0.001$ *vs* control, un-stimulated monocytes; $^{\circ\circ}P < 0.01$ *vs* PMA-stimulated monocytes.

Table 1
PMA-evoked superoxide anion production in human monocytes: effects of NCX 6550, pravastatin and SNP.

	% inhibition of PMA-evoked O_2^- production
NCX 50 μM	82 ± 4
NCX 10 μM	68 ± 4
NCX 1 μM	32 ± 2
PRAVA 50 μM	55 ± 6*
PRAVA 10 μM	30 ± 5*
PRAVA 1 μM	5 ± 3*
SNP 50 μM	32 ± 6*
SNP 10 μM	21 ± 5*
SNP 1 μM	6 ± 4*

Human monocytes were stimulated by PMA 10^{-6} M for 30 min, in the absence or presence of NCX 6550 (NCX), pravastatin (PRAVA) or sodium nitroprusside (SNP). PMA-evoked O_2^- production amounted to 59 ± 3 nmoles cytochrome *C* reduced/10^6 cells/30 min ($n = 4$). Values are mean ± S.E.M.; $n = 4$.

* $P < 0.05$ *vs* corresponding NCX concentrations.

We have also checked the ability of NCX 6550 to affect superoxide anion (O_2^-) production, since human monocyte/macrophages are major phagocytes and release relevant amounts of oxy-radicals upon challenge with appropriate stimuli. We confirm that the murine macrophage cell line does not undergo a significant respiratory burst, in contrast to human monocytes (Fig. 1B). As expected, a robust O_2^- production (59 ± 3 nmoles cytochrome *C* reduced/10^6 cells/30 min; $n = 4$) was documented in PMA-stimulated human monocytes, but not in LPS-challenged cells (Fig. 1B). Interestingly, NCX 6550 at 50 μM potently reduced (about 80% inhibition) PMA-induced O_2^- production (Fig. 1B; Table 1). When used at 50 μM, pravastatin also inhibited (by about 55%) PMA-induced respiratory burst in human monocytes, whereas SNP, at the same concentration, showed less effects on PMA-induced O_2^- generation (Table 1). Interestingly, at 1 μM concentration, NCX (but not the two other compounds) still inhibited PMA-induced O_2^- generation (Table 1).

3.2. NCX 6550 inhibits the release of pro-inflammatory cytokines in human monocyte/macrophages

According to previous experiments [20,21], a 24 h challenge with PMA (10^{-7} M) induces the maximal release of pro-inflammatory cytokines from human monocytes and MDM. PMA-evoked TNF-α release was higher in monocytes (1323 ± 58 pg/ml) than in MDM (574 ± 113 pg/ml; $n = 8$; $P < 0.05$ *vs* monocytes).

As depicted in Fig. 2, NCX 6550 and the PPARγ ligand PGJ inhibited, in a concentration-dependent manner (1 nM–50 μM), PMA-induced TNF-α release in both monocytes (Fig. 2A) and MDM (Fig. 2B). NCX 6550 was less potent than PGJ either in monocytes (Fig. 2A) with IC_{50} values of 240 and 92 nM, respectively, or in MDM (Fig. 2B), with IC_{50} values of 52 and 48 nM, respectively. Interestingly, the maximal inhibition (about 90%) afforded by NCX 6550 at 50 μM was significantly higher ($P < 0.001$) compared to that of pravastatin and the two NO donors, SNP and ISMN, used at the same concentration (Fig. 2).

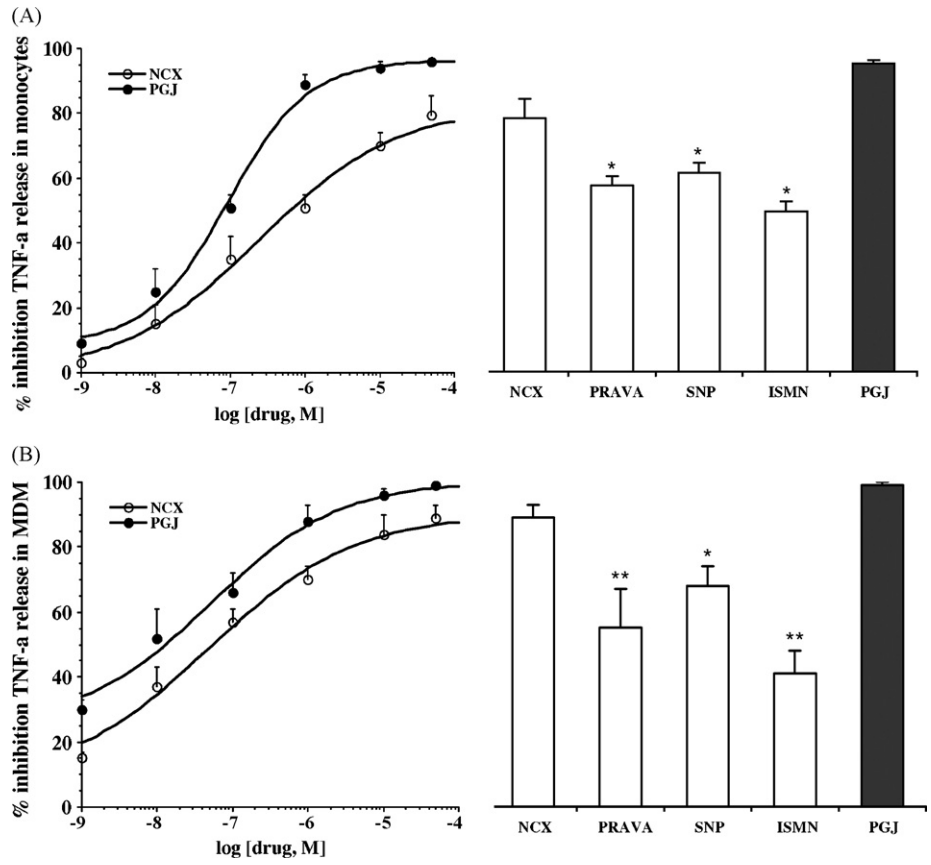

Fig. 2. NCX 6550 dose-dependently inhibits PMA-induced TNF-α release in human monocytes (A) and MDM (B). On the left: cells were pre-treated for 1 h with PGJ (1 nM–50 μM; ●) or NCX (1 nM–50 μM; ○) and then stimulated by PMA 10^{-7} M for 24 h. On the right: inhibition afforded at the maximal 50 μM concentration by NCX, pravastatin (PRAVA), sodium nitroprusside (SNP), isosorbide mononitrate (ISMN) or PGJ. Values are mean ± S.E.M.; $n = 8$. $^{*}P < 0.05$ *vs* NCX; $^{**}P < 0.01$ *vs* NCX.

Similar results were obtained with IL-6. PMA-induced IL-6 release was 468 ± 70 pg/ml ($n = 8$) in monocytes and 309 ± 28 pg/ml in MDM ($n = 8$; $P < 0.05$ *vs* monocytes). As shown in Fig. 3, NCX 6550 inhibited, in a concentration-dependent manner, PMA-induced IL-6 release in both human monocytes (Fig. 3A) and MDM (Fig. 3B), with IC_{50} values of 122 nM (monocytes) and 185 nM (MDM). Also for this marker, PGJ was more potent than NCX 6550 (IC_{50} values: 72 nM in monocytes and 89 nM in MDM). At the maximal 50 μM concentration, NCX 6550 was significantly more effective than pravastatin, SNP and ISMN (Fig. 3).

NCX 6550, as well as the other drugs, had no significant effect on PMA-induced IL-10 release (data not shown). In these experiments, PMA 10^{-7} M released small amounts of IL-10 (50 ± 7 pg/ml in monocytes and 45 ± 8 pg/ml in MDM; $n = 8$), significantly lower than the levels of pro-inflammatory cytokines.

3.3. NCX 6550 inhibits PMA-induced NF-κB translocation in human monocyte/macrophages

In these experiments, cells were pre-treated with drugs for 30 min and then stimulated by PMA (10^{-6} M) for 1 h, that ensures maximal effects [23]. In human monocytes, PMA induced a marked NF-κB nuclear translocation, with a major involvement of the p65 subunit, as demonstrated by supershift assays (Fig. 4A). NCX 6550, at 50 μM, potently inhibited PMA-induced NF-κB activation, whereas pravastatin was almost ineffective; SNP also reduced PMA-induced NF-κB activation (Fig. 4A). Moreover, NCX 6550 (10 nM–50 μM) produced a concentration-dependent inhibition of PMA-triggered NF-κB translocation in human monocytes (Fig. 4B). The endogenous PPARγ agonist PGJ, known to inhibit NF-κB activation [25], was also tested for comparison (Fig. 4B). Likewise, in human MDM, NCX 6550 and NO donors, but not pravastatin, inhibited PMA-induced NF-κB translocation (Fig. 4C).

3.4. NCX 6550 enhances PPARγ expression in human monocyte/macrophages

Since PPARγ is expressed in human monocyte/macrophages [16,17,20] and NO has been reported to increase PPARγ activity [26], we evaluated whether NCX 6550 affected PPARγ protein expression in human monocyte/macrophages.

In keeping with previous results [16,17,20], a lower constitutive expression of PPARγ protein was detected in human monocytes (PPARγ/β-actin ratio: 0.21 ± 0.031; $n = 4$; Fig. 5A) compared to MDM (PPARγ/β-actin ratio: 1.78 ± 0.38; $n = 4$; $P < 0.001$ *vs* monocytes; Fig. 5B). NXC 6550 and SNP, in the range of 1–50 μM, significantly enhanced PPARγ expression in monocytes, whereas pravastatin and ISMN were ineffective (Fig. 5A). On the contrary, the effects obtained in MDM were not statistical significant, possibly due to the higher constitutive PPARγ expression in these cells (Fig. 5B). In fact, even the PPARγ agonist PGJ (10 μM), tested as positive control, was less effective in MDM than in monocytes (Fig. 5).

By the Real-Time PCR, we also evaluated the effects of NCX 6550 on PPARγ gene expression in monocytes, in comparison with pravastatin, SNP, ISMN and two different PPARγ agonists, the endogenous ligand PGJ and the oral anti-diabetic drug rosiglitazone (Table 2). All drugs enhanced PPARγ mRNA levels of about twofold ($P < 0.05$ *vs* control monocytes; Table 2), NCX 6550 resulting particularly effective.

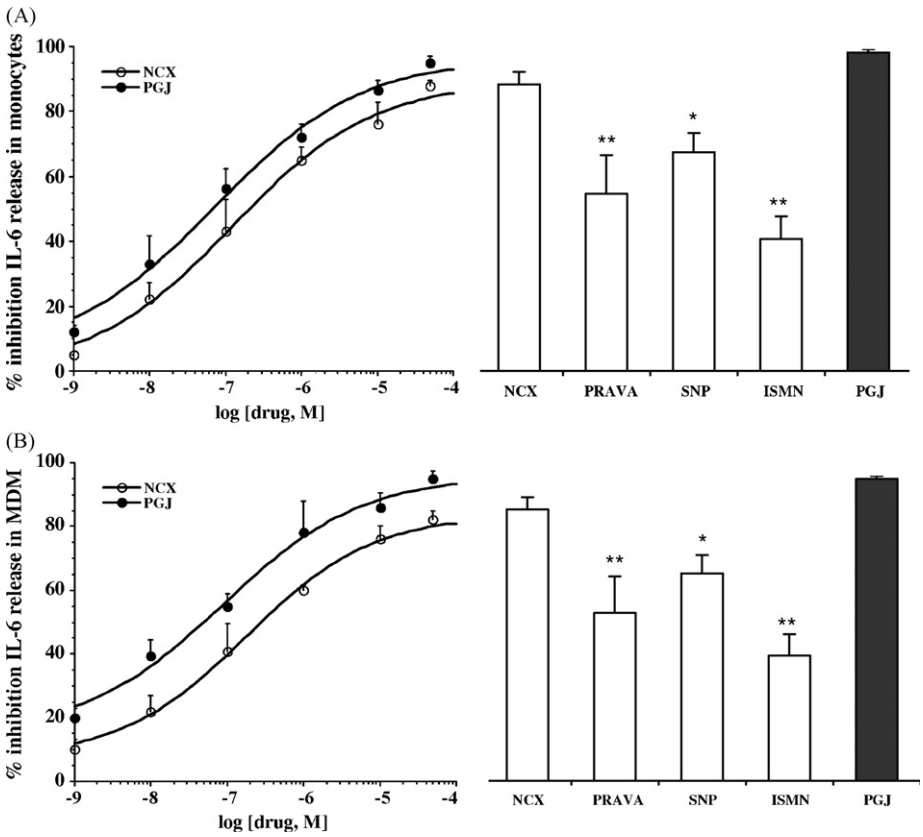

Fig. 3. NCX 6550 dose-dependently inhibits PMA-induced IL-6 release in human monocytes (A) and MDM (B). On the left: cells were pre-treated for 1 h with PGJ (1 nM–50 μM; ●) or NCX (1 nM–50 μM; ○) and then stimulated by PMA 10^{-7} M for 24 h. On the right: inhibition afforded at the maximal 50 μM concentration by NCX, pravastatin (PRAVA), sodium nitroprusside (SNP), isosorbide mononitrate (ISMN) or PGJ. Values are mean ± S.E.M.; $n = 8$. $^{*}P < 0.05$ *vs* NCX; $^{**}P < 0.01$ *vs* NCX.

Table 2
PPARγ gene expression in monocytes.

	Fold increase *vs* control (control = 1)	P^{*} value
Control	1	
PGJ	1.61 ± 0.05	0.05
ROSI	1.91 ± 0.04	0.05
NCX	2.01 ± 0.12	0.05
ISMN	1.68 ± 0.03	0.05
SNP	1.91 ± 0.06	0.05
PRAVA	1.97 ± 0.02	0.05

Monocytes were challenged for 2 h with PGJ (10 μM), rosiglitazone (ROSI; 1 μM), NCX (50 μM), pravastatin (PRAVA; 50 μM), SNP (50 μM) or ISMN (50 μM). mRNA from un-stimulated (control) monocytes ($n = 4$) represents the 'calibrator' (as called by the 7000 System JDS Software, Applied Biosystem) value = 1. Values are mean ± S.E.M.; $n = 4$.

* $P = 0.05$ *vs* control, un-stimulated monocytes. No significant differences among the different group treatments.

4. Discussion

The main finding of this study is that the NO-donating pravastatin, NCX 6550, inhibits PMA-induced cytokine release and NF-κB activation in human monocyte/macrophages and, at the highest concentration used (50 μM), enhances PPARγ expression in monocytes. Conversely, under the same experimental conditions, pravastatin (the native compound) and two reference NO donors, SNP and ISMN, do not display similar level of activity. It is worth noting that NCX 6550, a compound with a dual mode of action, i.e., inhibition of HMG CoA reductase and NO donation, maintains HMG CoA reductase inhibition properties, since, in rat aortic smooth muscle cells, it inhibits cholesterol biosynthesis with an IC_{50} value (9.4 μM: NicOx, internal data) in the same low micromolar range as reported with other NO-donating statins [27].

Overall, NCX 6550 shows its anti-inflammatory properties in a cell model, as human monocytes, that is relevant to atherosclerosis and coronary artery disease. In fact, the earliest noticeable lesions of atherosclerosis, fatty streaks, contain large numbers of foam cells, derived from circulating monocytes. After recruitment, monocytes adhere to the activated endothelium and migrate into the artery wall, where they differentiate into macrophages and accumulate lipids to form foam cells, which largely affect the progression of atherosclerosis. Indeed, not only they mediate the uptake of oxidized low-density lipoproteins (LDL), but also promote LDL oxidation, undergo a respiratory burst, release pro-inflammatory cytokines and secrete matrix metalloproteinases [28].

Moreover, human monocyte/macrophages are intriguing cell types to evaluate the NO involvement in inflammatory responses. Different experimental data of the early 1990s clearly point out that, at variance from rodent macrophages and murine cell lines, human monocyte/macrophages release little or no NO after *in vitro* challenge with standard stimuli, yet they express the iNOS gene and respective protein [5,6]. The lack of biopterin [6] and/or a different arginine transport-specific response [29] in human monocyte/macrophages could explain this discrepancy. Moreover, Carreras et al. [30] clearly showed the different kinetics of NO, oxy-radical and peroxynitrite production during the respiratory burst of human neutrophils.

Our results are consistent with these previous data, as we demonstrate a low but significant nitrite accumulation and a high superoxide anion production in PMA-stimulated monocytes. Interestingly, a 30-min challenge of human monocytes with NCX 6550 results in a low, although significant, increase in nitrite

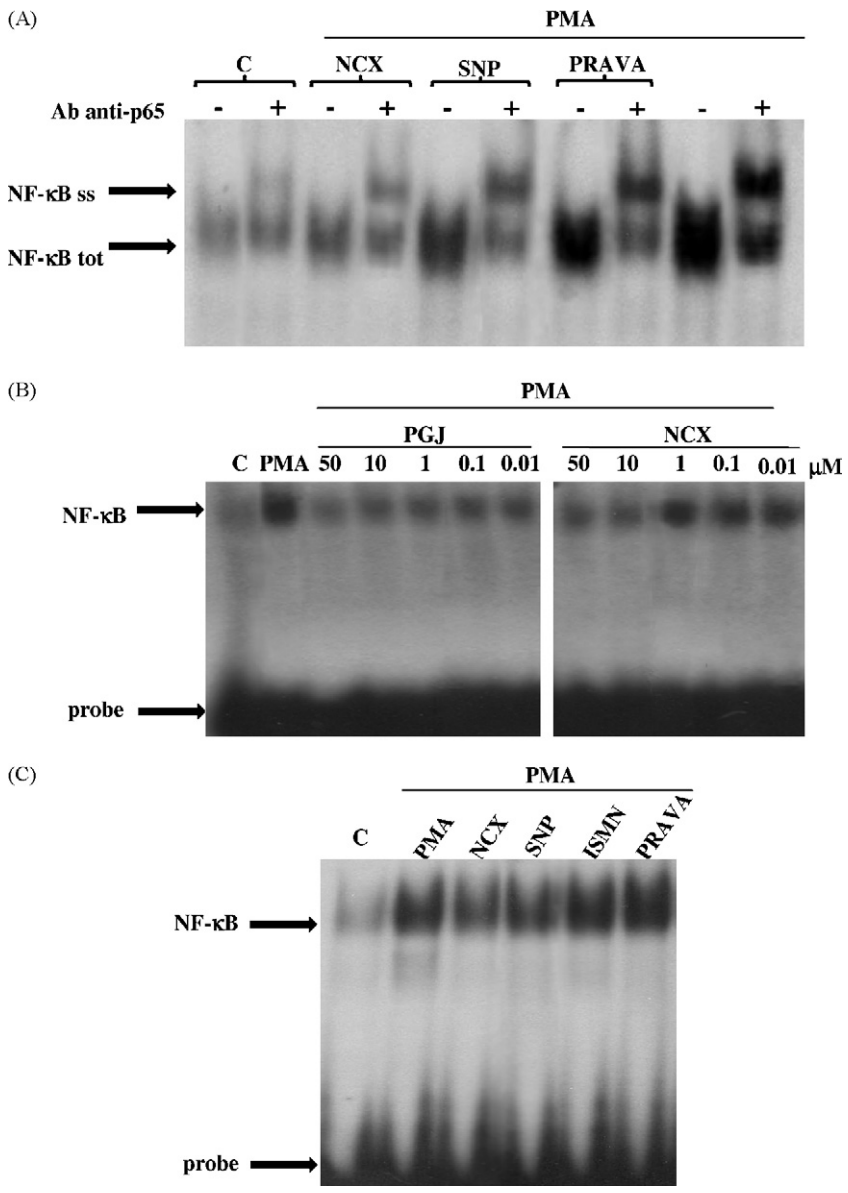

Fig. 4. NCX 6550 inhibits PMA-induced NF-κB translocation in human monocytes and MDM. In A: Effects of NCX 6550 and other compounds in human monocytes. Cells were pre-treated for 30 min with NCX, sodium nitroprusside (SNP), or pravastatin (PRAVA), all at 50 μM, and then stimulated by PMA 10^{-6} M for 1 h. Supershift (NF-κB ss) for p65 subunit is demonstrated. In B: NCX 6550 and PGJ inhibit PMA-induced NF-κB translocation in human monocytes in a concentration-dependent manner. Cells were pre-treated for 30 min with PGJ (10 nM–50 μM) or NCX (10 nM–50 μM), and then stimulated by PMA 10^{-6} M for 1 h. In C: effects of NCX 6550 and other compounds in human MDM. Cells were pre-treated for 30 min with NCX, pravastatin (PRAVA), sodium nitroprusside (SNP) or isosorbide mononitrate (ISMN), all at 50 μM, and then stimulated by PMA 10^{-6} M for 1 h. Each blot is representative of three other independent experiments.

accumulation, that further validates its NO-donating properties. Even more relevant is the NCX 6550 ability to inhibit, in a concentration-dependent manner, superoxide anion production in PMA-stimulated monocytes, thus confirming previous *ex vivo* data in splenocytes from both C57BL/6 and ApoE$^{-/-}$ mice [9]. NCX 6550 is significantly more effective than the native pravastatin and SNP: as reported here, at the highest 50 μM concentration, it inhibited PMA-induced O_2^- production by more than 80% and still displayed a 30% inhibition at 1 μM. On the contrary, neither pravastatin nor SNP exerted significant inhibition at 1 μM concentration.

Over the past years, several studies demonstrated that the pleiotropic effects of statins contribute to their therapeutic efficacy, beyond lipid lowering [13]. In particular, statins have been shown to inhibit NF-κB binding activity in LPS-stimulated human monocytes [14] and in TNF-α-challenged endothelial cells [31], as well as to activate PPARγ in murine macrophage cell lines [15]. Even more relevant, in patients with coronary artery disease, statins exert vascular protection and significantly improve endothelial function [32], possibly through mechanisms involving the NO pathway.

NO is a key mediator of endothelial function and statins are shown to enhance endothelial NO bioavailability by promoting NO production [33] and/or preventing its inactivation by free radicals [34]. NO has been shown to have a dual action on iNOS expression, that largely depends on the concentration that is released in a specific tissue, low physiological concentrations of NO inhibiting iNOS expression through a negative feedback mechanism [35]. Therefore, the incorporation of a NO-releasing moiety into a statin structure, and its slow release, as for NCX 6550, has the potential to confer further anti-inflammatory properties to the native molecule, besides increasing its liposolubility as previously demonstrated [4]. In fact, in other cell types, Ongini et al. [4] showed an increase of the theoretical partition coefficients (Log *P*, as a mea-

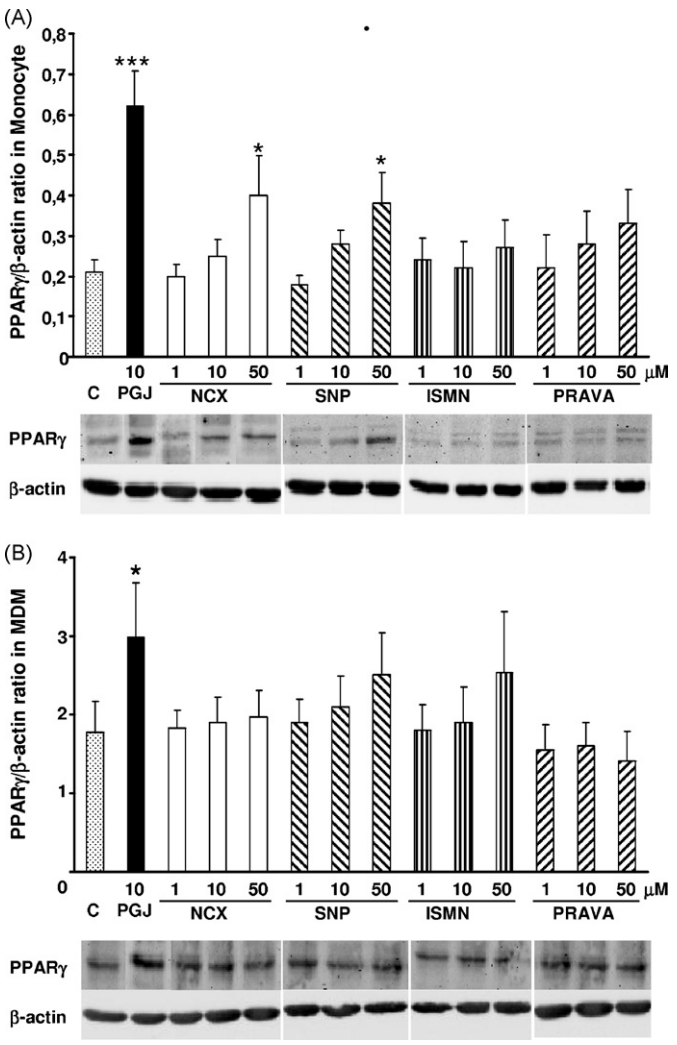

Fig. 5. PPARγ protein expression in human monocytes (A) and MDM (B). Semi-quantitative evaluation of PPARγ protein and representative Western blots for PPARγ and β-actin are shown. Cells were challenged for 6 h with the PPARγ ligand PGJ (10 μM), NCX (1–50 μM), sodium nitroprusside (SNP; 1–50 μM), isosorbide mononitrate (ISMN; 1–50 μM) or pravastatin (PRAVA; 1–50 μM). C denotes control, un-stimulated cells. Values are mean ± S.E.M.; $n = 4$. $^{*}P < 0.05$ *vs* control cells; $^{***}P < 0.001$ *vs* control cells.

sure of lipophilicity), pravastatin and NCX 6550 being 2.5 and 4, respectively.

The results here reported corroborate the above mentioned notions, since, in human monocyte/macrophages, NCX 6550 is more effective than pravastatin in inhibiting PMA-evoked release of TNF-α and IL-6, major biomarkers of cardiovascular risk [36]. As shown, NCX 6550 produced a concentration-dependent inhibition of cytokine release, being more potent in macrophages (IC_{50} value for TNF-α inhibition: 51 nM) than in monocytes (IC_{50} value: 240 nM), possibly due to a lower PMA-stimulated cytokine release in MDM. Interestingly, NCX 6550 appears to be more effective in human monocytes/MDM as compared to murine cells: the IC_{50} values found here are lower than those reported in RAW 264.7 cells (27.7 μM) and PC12 cells (2.3 μM) [4].

Release of pro-inflammatory cytokines is generally the result of gene transcription, which is controlled by the activation of various transcription factors. Among the different signal transduction pathways involved in cytokine secretion, we focused our attention on NF-κB and PPARγ, functionally active in human monocyte/macrophages [16,17,21]. Regulation of TNF-α production is largely NF-κB-dependent, although evidence exists that TNF-α and other cytokines can also be induced through NF-κB-independent pathways [21]; on its own, TNF-α is a potent inducer of NF-κB activation [21,31], as other inflammatory cytokines and oxy-radicals. So, an auto-regulatory cross-talk can occur, that is further tightly modulated by several mediators. As an example, NO can inhibit NF-κB through the induction and stabilization of its inhibitor, IκBα [37]. In addition, endogenous and exogenous PPARγ ligands have been repeatedly demonstrated to inhibit the release of pro-inflammatory cytokines [16–18] and NF-κB signalling pathway [25,38]. Our results are in keeping with the framework reported above. In fact, in both human monocytes and MDM, NCX 6550 inhibits, in a concentration-dependent manner, PMA-induced NF-κB translocation, with maximal inhibition at 50 μM. As expected from previous reports [25], PGJ, the endogenous agonist for PPARγ, is highly effective in inhibiting PMA-induced NF-κB translocation and more potent than NCX 6550; the maximal inhibition is achieved at 50 μM for both compounds and is significantly superior to that shown by the reference NO donors. Moreover, in these experiments, pravastatin is ineffective, at variance from previous data in human monocytes [39]. This discrepancy can be, at least in part, related to the different experimental procedures. In fact, Zelvyte et al. [39] used either un-stimulated monocytes or monocytes stimulated by oxidized low-density lipoproteins, and a longer incubation time (24 h) with pravastatin, compared to the present experiments (30 min).

Another interesting finding of the present study is that NCX 6550 significantly enhances PPARγ protein expression (about twofold increase) and mRNA levels in human monocytes, with a profile similar to that of PGJ. On the contrary, while effective at the mRNA level, pravastatin, SNP and ISMN do not induce a significant increase in PPARγ protein. Even if the effect of NCX 6550 is observed only at the highest (50 μM) concentration, it could contribute to its anti-inflammatory and anti-atherosclerotic potential, also in keeping with the suggested cross-talk between NO and PPARγ.

In fact, Ptasinska et al. [26] demonstrated that NO activates PPARγ signalling in endothelial cells, supporting, at least in part, the shared vascular protective properties of NO and PPARγ. Interestingly, Gonon et al. [40] reported that the cardio-protective effects of the PPARγ agonist rosiglitazone are mediated by NO. Moreover, in patients with type 2 diabetes, 2-week treatment with rosiglitazone reduced superoxide anion production, restored NO bioavailability and improved the *in vivo* re-endothelialization capacity of endothelial progenitor cells [41].

Therefore, the major ability of NCX 6550 to induce PPARγ protein expression, as well as its capability to inhibit PMA-induced cytokine release, oxy-radical production and NF-κB translocation, compared to the native pravastatin and reference NO donors, clearly indicate that this NO-donating statin might have relevant beneficial effects for the treatment of cardiovascular inflammatory disorders, such as atherosclerosis.

5. Conclusions

Altogether, these findings indicate that NCX 6550 is significantly more effective than pravastatin, SNP and ISMN and suggest this compound as a potent regulator of human monocyte/macrophage activity. NCX 6550 anti-inflammatory effects result in a significant reduction of PMA-evoked respiratory burst and cytokine release, as well as inhibition of NF-κB translocation and stimulation of PPARγ expression. We suggest that, in human monocyte/macrophages, the anti-inflammatory potential of this compound is determined, at least in part, by its NO-donating properties, since the effects exerted by NCX 6550 are significantly higher than those evoked by the native pravastatin. These data further indicate that the incorporation of a NO-donating moiety into a statin structure confers

pharmacological properties which may translate into useful therapeutic benefits.

Conflict of interest

Ennio Ongini is employed by the NicOx Research Institute (Via Ariosto 21, 20091 Bresso, Milan, Italy), the company that develops NCX 6550, and holds stock in the NicOx company. All the other authors state no conflict of interest.

Acknowledgements

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PAPER (2)

"Autocrine activation of human monocyte/macrophages by monocyte-derived microparticles and modulation by PPARγ ligands"

Bardelli C, Amoruso A, Federici Canova D, Fresu L, Balbo P, Neri T, Celi A, Brunelleschi S

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Several studies in the last decade dealt with microparticles (MPs), underlining their biological importance as carrier of bioactive effectors, actively involved in physiological and patho-physiological processes. Monocytes can release MPs upon activation with different stimulus [35]. It has been previously demonstrated that monocyte-derived MPs can affect endothelial cells [101] and human bronchial epithelial and alveolar cell lines [56,57]. In human airway cells, they up-regulate the synthesis of IL-8, CCL-2 (chemokine C-C motif ligand-2) and ICAM-1, through NF-κB activation and PPAR-γ modulation [57]. This study aimed to evaluate the ability of monocyte-derived MPs to stimulate human monocytes and MDM, representing the first one dealing with a possible autocrine effect of these MPs. We first demonstrated that monocyte-derived MPs induce a concentration-dependent superoxide anion production and TNF-α and IL-6 release in monocytes and monocyte-derived macrophages (MDM). These effects were reduced in presence of PPAR-γ agonists, 15d-PGJ and rosiglitazone, and reverted by the selective PPAR-γ antagonist GW9662, confirming the direct involvement of the receptor. Monocyte-derived MPs were also able to induce NF-κB nuclear translocation, which again was significantly reduced by PPAR-γ agonists, in agreement with previous reports [57,102]. Moreover, monocyte-derived MPs enhanced PPAR-γ protein expression in both human monocytes and MDM. In this study therefore, we demonstrated that in human monocytes/macrophages, monocyte-derived MPs exert an autocrine activation that was modulated by PPAR-γ ligands. MPs had relevant pro-inflammatory effects, which were reduced by PPAR-γ agonist. On the other hand, these data indicate, for the first time, that MPs can enhance PPAR-γ expression, representing a possible anti-inflammatory property that sustains and extends previous data describing the anti-inflammatory effects of MPs [31,60,103].

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RESEARCH PAPER

Autocrine activation of human monocyte/macrophages by monocyte-derived microparticles and modulation by PPARγ ligands

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Monocyte-derived microparticles; human monocytes; monocyte-derived macrophages; oxy-radical production; inflammatory cytokines; NF-κB; PPARγ; rosiglitazone

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BACKGROUND AND PURPOSE

Microparticles (MPs), small membrane-bound particles originating from different cell types during activation or apoptosis, mediate intercellular communication, exert pro-coagulant activity and affect inflammation and other pathophysiological conditions. Monocyte-derived MPs have undergone little investigation and, to our knowledge, have never been evaluated for their possible autocrine effects. Therefore, we assessed the ability of monocyte-derived MPs to stimulate human monocytes and monocyte-derived macrophages (MDM).

EXPERIMENTAL APPROACH

MPs were generated from supernatants of human monocytes stimulated by the calcium ionophore A23187 (12 μM), and then characterized. Human monocytes and MDM of healthy donors were isolated by standard procedures. Cells were challenged by MPs or phorbol 12-myristate 13-acetate (PMA, used as standard stimulus), in the absence or presence of PPARγ agonists and antagonists. Superoxide anion production (measured spectrophotometrically), cytokine release (ELISA), PPARγ protein expression (immunoblotting) and NF-κB activation (EMSA assay) were evaluated.

KEY RESULTS

Monocyte-derived MPs induced, in a concentration-dependent manner, oxygen radical production, cytokine release and NF-κB activation in human monocytes and macrophages, with lower effects than PMA. In both cell types, the PPARγ agonists rosiglitazone and 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2) inhibited MPs-induced stimulation and this inhibition was reversed by a PPARγ antagonist. In human monocyte/macrophages, MPs as well as rosiglitazone and 15d-PGJ_2 induced PPARγ protein expression.

CONCLUSION AND IMPLICATIONS

In human monocyte/macrophages, monocyte-derived MPs exert an autocrine activation that was modulated by PPARγ ligands, inducing both pro-inflammatory (superoxide anion production, cytokine release and NF-κB activation) and anti-inflammatory (PPARγ expression) effects.

Abbreviations

GW9662, 2-chloro-5-nitro-N-phenylbenzamide; ICAM, intercellular adhesion molecule; MDM, monocyte-derived macrophages; MPs, microparticles; 15d-PGJ_2, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2; PMA, phorbol 12-myristate 13-acetate; PS, phosphatidylserine; PSGL, P-selectin glycoprotein ligand-1; ROS, reactive oxygen species; TF, tissue factor

Introduction

Microparticles (MPs), also referred to as microvesicles, are small (diameter 0.1–1 μm) membrane-bound particles that originate from the cell surface of most (if not all) cell types during activation, shear stress or apoptosis. MPs are heterogeneous in nature, varying in both size and content, and present cell surface markers and cytoplasmic components of the parent cells from which they originate. Therefore, surface markers are largely used to identify the derivation of MPs. As an example, CD14 is the major marker for monocyte-derived MPs, whereas CD3, CD41a and CD146 are markers for lymphocyte-, platelet- and endothelial cell-derived MPs respectively (Dalli *et al.*, 2008).

In addition to cell-specific markers, MPs carry a broad variety of proteins, nucleic acids and lipids, that enable them to mediate intercellular communication (thereby acting as novel signalling elements), to transfer genetic information (due to their content of nucleic acids and proteins), to exert pro-coagulant activity and to affect inflammation, as well as other pathophysiological conditions (Mallat *et al.*, 1999; Hugel *et al.*, 2004; Distler *et al.*, 2005b; Ratajczak *et al.*, 2006; Dalli *et al.*, 2008; Beyer and Pisetsky, 2010). For these reasons, MPs have been the focus of intense experimental research in the last decade and are now recognized to play differing roles, depending on the cell of origin.

Although present at low levels (5–50 $\mu g \cdot mL^{-1}$; Ratajczak *et al.*, 2006) in the blood from healthy donors, markedly increased numbers of circulating MPs have been detected under different pathological conditions, such as atherosclerosis, diabetes, sepsis, arthritis and cancer (Mallat *et al.*, 2000; Nieuwland *et al.*, 2000; Distler *et al.*, 2005b; Ratajczak *et al.*, 2006; Anderson *et al.*, 2010; Beyer and Pisetsky, 2010). The majority (about 80%) of *in vivo* circulating MPs derive from platelets (Ratajczak *et al.*, 2006) and most experimental work so far performed deals with platelet-derived MPs. As an example, platelet-derived MPs enhance neutrophil aggregation and adhesion (even when L-selectin is blocked), deliver arachidonic acid to other cells, and increase monocyte chemotaxis and/or adhesion to the endothelium (Barry *et al.*, 1998; Distler *et al.*, 2005a). Moreover, neutrophil-derived MPs induce the expression of IL-6 and the chemokine CCL2 (MCP-1) in endothelial cells (Mesri and Altieri, 1999), but, under certain circumstances, can also exert anti-inflammatory effects (Gasser and Schifferli, 2004; Hugel *et al.*, 2004; Dalli *et al.*, 2008).

Although less thoroughly investigated, monocyte-derived MPs also play a key role in haemostasis and thrombosis. In fact, they express tissue factor (TF) at high levels, in addition to P-selectin glycoprotein ligand-1 (PSGL-1). Therefore, these MPs not only directly activate platelets (*via* PSGL-1), but also fuse with them and transfer TF to platelet membranes, thereby promoting optimal coagulation by activated platelets (Del Conde *et al.*, 2005). Recently, Li *et al.* (2010) demonstrated that exposure of THP-1 cells or human monocyte-derived macrophages (MDM) to tobacco smoke extract induces the generation of highly pro-coagulant MPs that contribute to the pathological hypercoagulability of smokers. Moreover, atherosclerotic plaques have been shown to contain pro-coagulant MPs (Mallat *et al.*, 1999) that are mainly derived from monocyte/macrophages, as demonstrated by co-labelling for CD14 and IgG (>90% of IgG containing MPs is $CD14^+$; Mayr *et al.*, 2009). In addition, plaque-derived MPs have been shown to transfer the adhesion molecule ICAM-1 to endothelial cells, thus promoting monocyte adhesion and transendothelial migration (Rautou *et al.*, 2011).

Evidence is also emerging that monocyte-derived MPs affect endothelial cells (Essayagh *et al.*, 2007; Aharon *et al.*, 2008) as well as human bronchial epithelial and alveolar cell lines (Cerri *et al.*, 2006; Neri *et al.*, 2011). In human airway cells, monocyte-derived MPs have been shown to up-regulate the synthesis of IL-8, CCL2 and ICAM-1 (Cerri *et al.*, 2006). Moreover, MPs-induced pro-inflammatory effects are mediated by NF-κB activation and modulated by PPARγ agonists (Neri *et al.*, 2011). This is an interesting finding, as activation of PPARγ (expressed in a large variety of cells, including human airway cells and monocyte/macrophages) by selective agonists results in consistent anti-inflammatory effects (Jiang *et al.*, 1998; Ricote *et al.*, 1998; Amoruso *et al.*, 2007, 2008, 2010; Neri *et al.*, 2011).

To our knowledge, there is no information concerning a possible autocrine effect of monocyte-derived MPs. Therefore, we evaluated the ability of monocyte-derived MPs to stimulate human monocytes and monocyte-derived macrophages (MDM).

In this study we demonstrate that monocyte-derived MPs induce, in a concentration-dependent manner, oxygen radical production, cytokine release, NF-κB activation and PPARγ protein expression in human monocytes and MDM. Moreover, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2) and rosiglitazone, two PPARγ agonists which exert relevant anti-inflammatory activity in human monocyte/macrophages (Jiang *et al.*, 1998; Amoruso *et al.*, 2007, 2008, 2010), modulate MPs-induced effects.

Methods

Generation of monocyte-derived MPs and MPs characterization

MPs were generated according to Neri *et al.* (2011). Briefly, human monocytes (about 2×10^6 cells per well; see below for monocyte isolation) were stimulated by the calcium ionophore A23187 (12 μM), in a final volume of 0.5 mL, for 20 min at 37°C. The supernatant was recovered and cleared by centrifugation (14 000× *g*, 5 min, room temperature) to remove cell debris.

Such supernatants from six individual non-smoker volunteers (mean age 34.6 + 2.9 yr, range 27-45 yr, 3 males, 3 females; no present medication) were used in these experiments and the MPs characterized by assessing $CD14^+$ elements, phosphatidylserine (PS) expression and TF activity.

PS-positive MPs in each sample were detected using the Zymuphen MP-activity kit (Hyphen BioMed, Neuville-sur-Oise, France), according to the manufacturer's instructions and expressed as PS equivalents; the PS concentration in supernatants was about 2 nM (see also *Results*). TF activity was measured by a one-stage clotting time as described by Celi *et al.* (1994). Briefly, 100 μL MPs were mixed with 100 μL normal human plasma at 37°C for 5 min; 100 μL of 25 mM

$CaCl_2$ was added to the mixture and the time to clot formation was recorded. The test tube was kept in a transparent water bath at 37°C throughout the test. The values were converted to arbitrary units (A.U.) of pro-coagulant activity by comparison with a standard curve obtained using a human brain thromboplastin standard; this preparation was assigned a value of 1000 A.U. for a clotting time of 20 s (Celi *et al.*, 1994).

To characterize the expression of CD14 in MPs generated from monocytes, samples were analysed by flow cytometry using a FACS Calibur (Becton Dickinson, Oxford, UK). Data acquisition and analysis were performed using WinMDi 2.9 software (Joseph Trotter, The Scripps Institute). To determinate the percentage of $CD14^+$ MPs, 100 µL of resuspended human monocytes and 100 µL of MPs were added for 30 min on ice to the fluorescent dye-labelled antibody against surface marker $CD14^+$ PE (Phycoerythrin)-conjugated (Dako Cytomation, Denmark). Incubation was performed in the dark and the expression of the surface marker was analysed. CD14 expression positivity was defined on FL2 (PE fluorescence) versus FSC dot plot representation. Regions (R1) corresponding to monocytes and MPs were defined on forward versus side angle light scatter intensity dot plot representation. The forward light scatter setting was E-01 and a total of 10 000 events was analysed.

Following procedures outlined by Scanu *et al.* (2008) and Carpintero *et al.* (2010), we also measured the total protein content in the supernatant samples; this allows a quantitative measure, as well as a better evaluation of possible concentration-dependent effects of MPs. The determination of protein content was measured using a bicinchoninic acid (BCA) assay: 100 µL of supernatant from A23187-challenged monocytes usually contained about 10 µg proteins (see also *Results*).

In order to ensure that the effects observed (see below) with our MPs preparations were not attributable to soluble mediators that may be present within supernatants, supernatants were ultracentrifuged at 100 000× *g* for 2 h. In this latter case, both supernatant (from which MPs have been cleared) and pellet (that contains MPs and is resuspended in the same volume as the starting material) were evaluated.

Preparation of monocytes and MDM

This study and the research protocol were approved by the Ethical Committee of the Azienda Ospedaliera Maggiore della Carità, Novara (Italy) and informed written consent was obtained from all participants.

Human monocytes were isolated either from fresh buffy coats, obtained from the local blood bank, or from heparinized venous blood (30–40 mL) of healthy non-smoker volunteers by the standard techniques of dextran sedimentation, Histopaque (density = 1.077 $g \cdot cm^{-3}$)-gradient centrifugation (400× *g*, 30 min, room temperature) and finally recovered by thin suction at the interface, as described (Amoruso *et al.*, 2007, 2008, 2009a). Cells were resuspended in RPMI 1640 medium, supplemented with 5% heat-inactivated foetal bovine serum (FBS), 2 mM glutamine, 10 mM HEPES, 50 $\mu g \cdot mL^{-1}$ streptomycin and 5 $U \cdot mL^{-1}$ penicillin. Purified monocyte populations were obtained by adhesion (2 h, 37°C, 5% CO_2), non-adherent cells (mainly lymphocytes) being gently removed with sterile PBS. Cell viability (Trypan blue dye exclusion) was usually >98%. MDM were prepared from monocytes, by culture (8–10 days) in RPMI 1640 medium containing 20% FBS, glutamine and antibiotics; MDM were defined as macrophage-like cells by evaluating surface markers CD14, MHCII, CD1a and CD68, as described (Amoruso *et al.*, 2008, 2009b). The effects of different treatments (see below) on cell viability were determined by the methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay. In these experiments, monocytes (1×10^5) were challenged with the compounds under study for different times (up to 24 h). Thereafter, the medium was replaced by the MTT solution (1 $mg \cdot mL^{-1}$) and cells were incubated for 2 h at 37°C in the dark. The supernatant was removed and DMSO was added in order to dissolve the purple formazan; the absorbance of the samples was read at 580 and 675 nm, as described (Amoruso *et al.*, 2010).

Different numbers of cells were used according to the type of experiments – 2×10^6 cells for Western blot experiments, 5×10^6 cells for EMSA assays, $0.5–1 \times 10^6$ cells for superoxide anion production and 1×10^6 cells for cytokine release.

Superoxide anion (O_2^-) production in monocytes and MDM

Adherent cells ($0.5–1 \times 10^6$ cells per well) were washed twice with PBS, incubated in RPMI 1640 medium (without phenol red, no antibiotics and no FBS) and challenged with different aliquots (1–300 µL, corresponding to 0.1 and 30 µg protein concentration respectively) of MPs-containing supernatants from A23187-stimulated monocytes for 30 min, in order to evaluate concentration-dependent effects. MPs effects were compared with those evoked by maximally effective concentrations of the PKC activator, phorbol 12-myristate 13-acetate (PMA; 1 µM). In some experiments, cells were pretreated with 15d-PGJ_2 (10 µM), an endogenous PPARγ agonist, for 30 min and then challenged with MPs or PMA. O_2^- production, evaluated by the superoxide dismutase (SOD)-inhibitable cytochrome C reduction, was expressed as nmol cytochrome C reduced per 10^6 cells per 30 min, using an extinction coefficient of 21.1 mM (Bardelli *et al.*, 2005). To avoid interference with spectrophotometric recordings of O_2^- production, human monocytes and MDM were incubated with RPMI 1640 without phenol red. Experiments were performed in duplicate; control values (e.g. basal O_2^- production in the absence of stimuli) were subtracted from all determinations.

Cytokine release in monocyte and MDM

Human monocytes and MDM were challenged with increasing concentrations of MPs or PMA (0.1 µM) for 24 h. This time period has been previously shown to ensure maximal cytokine release (Bardelli *et al.*, 2005; Amoruso *et al.*, 2010). Supernatants were collected and stored at –80°C. Cytokine content in the samples was measured using enzyme-linked immunoassay kit (R&D Systems, Minneapolis, USA).

TNF-α and IL-6 were evaluated as the most relevant pro-inflammatory cytokines; the amount of each cytokine was expressed in $pg \cdot mL^{-1}$, as indicated by the manufacturer's instructions. The minimum detectable concentrations of human TNF-α and IL-6 were 1.4 $pg \cdot mL^{-1}$ and 0.5 $pg \cdot mL^{-1}$ respectively. No cross-reactivity was observed with any other known cytokine. Control values (e.g. cytokine release from

untreated, un-stimulated cells) were subtracted from all determinations. In some experiments, cells were pretreated for 1 h with 15d-PGJ_2 (10 μM) or rosiglitazone (1 μM), in the absence or presence of the PPARγ antagonist 2-chloro-5-nitro-N-phenylbenzamide (GW9662; 1 μM), and then stimulated by MPs or PMA for 24 h. At the concentrations reported above, all the compounds had no relevant cytotoxicity (MTT assay; data not shown).

Evaluation of NF-κB activation

The activation of NF-κB induced by MPs or PMA was evaluated by measuring its nuclear migration (by EMSA), as previously described (Bardelli *et al.*, 2005). In EMSA assays, nuclear extracts (5 μg) from monocytes or MDM were incubated with 2 μg poly (dI-dC) and [^{32}P] ATP-labelled oligonucleotide probe (100 000–150 000 cpm; Promega) in binding buffer for 30 min at room temperature. The NF-κB consensus oligonucleotide (5′-AGTTGAGGGGACTTTCCCAGGC–3′) was from Promega. The nucleotide–protein complex was separated on a polyacrylamide gel, the gel was then dried and radioactive bands were detected by autoradiography (Bardelli *et al.*, 2005; Neri *et al.*, 2011). Densitometric analysis of NF-κB nuclear translocation was also performed, with values for un-stimulated cells being set to 1. In some experiments, cells were pretreated with 15d-PGJ_2 (10 μM) or rosiglitazone (5 μM) for 1 h and then challenged with MPs or PMA. At the concentrations used, neither 15d-PGJ_2 nor rosiglitazone exerted cytotoxic effects (evaluated by MTT assay; not shown).

PPARγ protein expression and semi-quantitative analysis

The constitutive expression of PPARγ protein was evaluated in monocytes and MDM, as described (Amoruso *et al.*, 2007, 2008). Cells (2×10^6), seeded in six-well plates, were washed twice with ice-cold PBS, scraped off in radioimmunoprecipitation assay buffer and lysed by sonication; when necessary, cell lysates were stored at –80°C. Protein concentration was measured by the BCA assay. Protein samples (20 μg) were analysed by SDS-PAGE (10% acrylamide) and electro-blotted on nitrocellulose membrane (Protran, Perkin Elmer Life Sciences, Boston, MA, USA). Immunoblots were performed using polyclonal rabbit anti-human PPARγ (Abcam, UK), that detects both PPARγ 1 and 2 isoforms, and monoclonal mouse anti-human β-actin (Sigma, Milan, Italy) antibodies; anti-mouse and anti-rabbit secondary antibodies were coupled to horseradish peroxidase. Chemiluminescence signals were analysed under non-saturating conditions with an image densitometer (Versadoc, Bio-Rad, Hercules, USA). Semi-quantitative evaluation of PPARγ protein was performed by calculating the ratio between its total expression (i.e. the sum of the two isoforms) and the expression of the reference housekeeping protein, β-actin (Amoruso *et al.*, 2007, 2008).

Statistical analysis

All statistical analyses were performed using SPSS statistical software (version 15.0, SPSS Inc., Chicago, IL, USA). Data are expressed as mean ± SEM of '*n*' independent experiments on cells isolated from different healthy donors; cytokine and oxy-radical determinations were performed in duplicate. Concentration–response curves for MPs were constructed and EC_{50} values were interpolated from curves of best-fit. Statistical evaluation was performed by ANOVA and Bonferroni correction or, when required, by unpaired, two-tailed Student's *t*-test. A non-parametric test (Mann–Whitney test) was used to evaluate the data transformed to percentage, as in the case of cytokine and O_2^- inhibition, and EMSA quantification. Differences were considered statistically significant when $P < 0.05$.

Materials

FBS was from Gibco (Paisley, UK). Rosiglitazone, GW9662 and 15d-PGJ_2 were from Cayman Chemicals (Milan, Italy). Histopaque, PBS, RPMI 1640 medium (with or without phenol red), glutamine, HEPES, streptomycin, penicillin, PMA, SOD, cytochrome C and monoclonal mouse anti-human β-actin antibody were obtained from Sigma (Milan, Italy). All the reagents for EMSA assays were purchased from Promega Corporation (St. Louis, USA). The polyclonal rabbit anti-human PPARγ antibody was from Abcam (UK). Tissue-culture plates were from Nunc Ltd (Denmark); all cell culture reagents, with the exception of FBS, were endotoxin-free according to details provided by the manufacturer. TNF-α and IL-6 immunoassay kits were obtained from R&D Systems (Minneapolis, USA). The Zymuphen MP-activity kit was purchased from Hyphen BioMed (Neuville-sur-Oise, France).

Results

Characterization of monocyte-derived MPs

The MPs used in this study are generated from A23187-challenged human monocytes (isolated from six healthy donors). In order to characterize these MPs, supernatants (100 μL) from un-stimulated or A23187-challenged monocytes were evaluated for CD14 expression, TF activity and PS equivalents (Figures 1 and 2). Figure 1 shows a forward versus side angle light scatter dot plot and $CD14^+$ expression of un-stimulated monocytes (A) and MPs (B). As expected, monocyte-derived MPs are smaller than monocytes, as demonstrated by physical parameters analysed by FACS. Since about 95% of our MPs preparation is $CD14^+$, we can conclude that they have a monocytic origin. Moreover, as shown in Figure 2, only supernatants from A23187-stimulated monocytes demonstrated significant TF activity and PS concentration, thus confirming MPs formation. In an attempt to quantify MPs and in view of their possible concentration-dependent effects, we have evaluated the total protein content of the supernatants from A23187-stimulated monocytes ($n = 6$), using different aliquots (1, 5, 10, 100 or 300 μL) containing 0.1, 0.5, 1, 10 and 30 μg protein respectively (data not shown). Since a final volume of 1 mL is used in all the experimental assays performed (see below), MPs were used in the range 0.1–30 μg protein mL^{-1}.

Monocyte-derived MPs induce oxygen radical production in human monocytes and MDM

Monocyte-derived MPs induce, in a concentration-dependent manner (0.1–30 μg protein mL^{-1}), O_2^- production in human monocytes and MDM (Figure 3A), with a maximal effect at

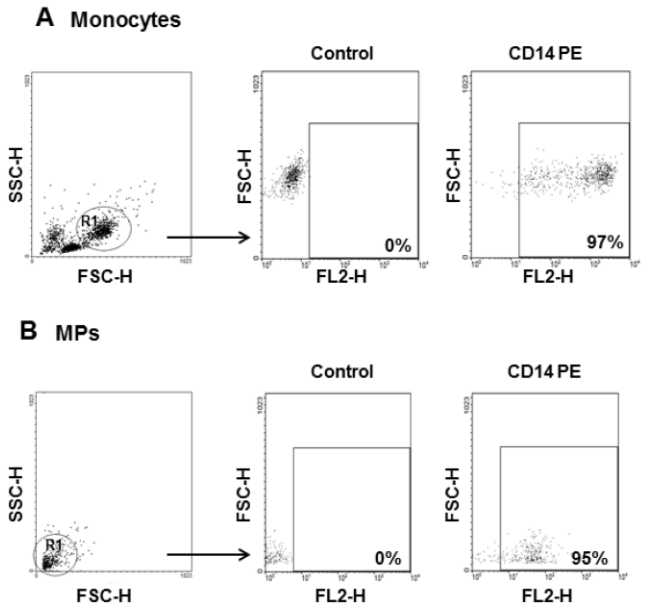

Figure 1

Flow cytometry analysis of monocyte-derived microparticles (MPs). Human monocytes (1×10^6; resuspended in 100 µL PBS) and 100 µL of MPs were analysed. Regions (R1) corresponding to monocytes (A) and MPs (B) were defined on forward (FSC) versus side angle light scatter (SSC) intensity dot plot representation and CD14 expression positivity was defined on FL2 (PE fluorescence) versus FSC dot plot representation. MPs have smaller physical parameters and show similar percentage of CD14 positivity compared to monocyte population.

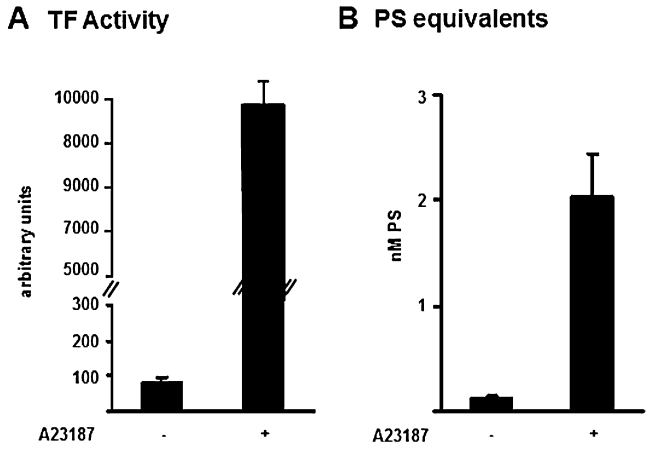

Figure 2

Characterization of monocyte-derived microparticles (MPs). The TF activity in supernatant (100 µL) from un-stimulated or stimulated (A23187, 12 µM) monocytes is shown in A and the corresponding concentrations of PS equivalents in B. Data are expressed as mean ± SEM of six separate MPs preparations. See text for further details.

10–30 µg·mL^{-1} and with EC_{50} values of 1.55 µg·mL^{-1} (monocytes) and 2 µg·mL^{-1} (MDM). As shown in Figure 3B, MPs-induced oxygen radical production is significantly lower than that evoked by PMA (1 µM). Moreover, spontaneous O_2^- production is minimal in both cell types, and ultra-centrifuged supernatants (from which MPs have been removed) or supernatants from un-stimulated human monocytes do not affect the respiratory burst. Conversely, the pellet from ultra-centrifuged samples induced O_2^- production, although less effectively that the original MPs. Interestingly, 4 µM A23187 (the concentration present in the 300 µL aliquot of supernatant) has minor effects (Figure 3B), and even at 12 µM evokes only a low respiratory burst (not shown). As expected, the endogenous PPARγ agonist, 15d-PGJ_2, used at 10 µM, potently inhibited PMA-induced O_2^- production: about 90–95% in both monocytes and MDM (Figure 3C). In human monocytes, the endogenous PPARγ agonist is less potent ($P < 0.01$) in inhibiting MPs-induced O_2^- production than PMA-evoked O_2^- production (Figure 3C). In keeping with previous observations (Amoruso *et al.*, 2008, 2009b), the PPARγ antagonist GW9662 completely abolishes the inhibitory effect of 15d-PGJ_2 on oxygen radical production (not shown).

Monocyte-derived MPs induce cytokine release in human monocytes and MDM

As shown in Figure 4, PMA and MPs induced TNF-α and IL-6 release in human monocytes and MDM. In both cell types, the phorbol ester evokes a significantly higher cytokine production than MPs. Furthermore, MPs, evaluated in the range 1–30 µg protein mL^{-1}, exerted concentration-dependent effects with maximal effects at 10 µg·mL^{-1} (protein concentration; Figure 4).

In human monocytes (Figure 4A), MPs released higher amounts of IL-6 than of TNF-α, whereas PMA-evoked release was not significantly different. Basal cytokine release from un-stimulated human monocytes (TNF-α = 10 ± 4 pg·mL^{-1} and IL-6 = 38 ± 14 pg·mL^{-1}; n = 5) was subtracted from all determinations. Maximal release by MPs of TNF-α or of IL-6 was observed at 10 µg·mL^{-1} protein concentration (Figure 4A) and was enhanced by the PPARγ antagonist, GW9662 (1 µM). Rosiglitazone (1 µM) and 15d-PGJ_2 (10 µM), previously shown to exert maximal effects with no evident sign of cytotoxicity (Amoruso *et al.*, 2008, 2009b, 2010), inhibited MPs-induced cytokine release (see also Table 1) and this inhibition was completely reversed by GW9662 (Figure 4A).

In human MDM (Figure 4B), PMA and MPs induced TNF-α and IL-6 release (in lower amounts than in human monocytes) that was potently inhibited by the two PPARγ ligands (15d-PGJ_2 exerting greater effects than rosiglitazone) . This inhibition was reversed by GW9662. As described previously, basal cytokine release (TNF-α = 20 ± 5 pg·mL^{-1}, IL-6 = 25 ± 10 pg·mL^{-1}; n = 5) is subtracted from all determinations.

As shown in Table 1, some differences are observed concerning the inhibitory potency of the two PPARγ agonists on cytokine release, which appears to depend on both the cell type and the stimulus. The inhibition afforded by PPARγ ligands is more evident in monocytes than in MDM (Table 1). In these experiments, the inhibition by rosiglitazone on MPs-induced cytokine release was usually less (although not always reaching statistical significance) than PMA-induced cytokine release. This effect was particularly evident in human monocytes, in which rosiglitazone inhibited by more than 60% the PMA-induced TNF-α release, while it reduced MPs-evoked release by only 27% (Table 1).

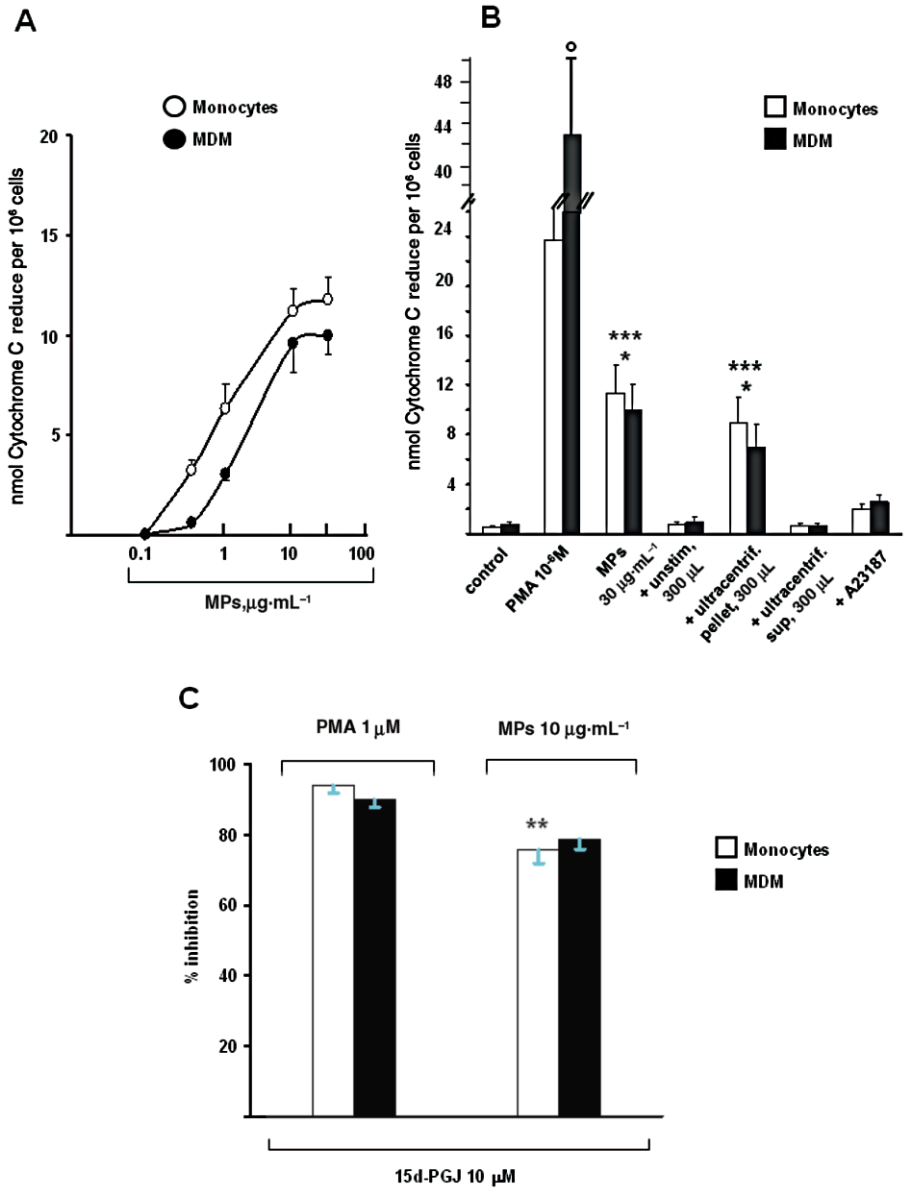

Figure 3

Monocyte-derived MPs induce superoxide anion (O_2^-) production in human monocytes and monocyte-derived macrophages (MDM). Human monocytes and MDM were challenged with the different stimuli for 30 min. In A: concentration-dependent (0.1–30 µg·mL^{-1}) effects of MPs. Values are means ± SEM; n = 14 (monocytes), 8 (MDM). In B: superoxide anion production induced by MPs, PMA, A23187, ultra-centrifuged supernatants and pellets. Cells were challenged with PMA (1 µM), MPs (30 µg·mL^{-1}), A23187 (4 µM), supernatant (300 µL) from un-stimulated monocytes, ultra-centrifuged supernatant (300 µL) from A23187-stimulated monocytes or ultra-centrifuged pellet (300 µL) from A23187-stimulated monocytes. Please, note that, in these experiments, spontaneous (control) O_2^- production was not subtracted. +unstim, 300 µL = supernatant (300 µL) from un-stimulated monocytes added to cells; +ultracentrif, sup 300 µL = ultra-centrifuged supernatant (300 µL) from A23187-challenged monocytes added to cells; +ultracentrif, pellet 300 µL = ultra-centrifuged pellet (300 µL) from A23187-challenged monocytes added to cells. Mean ± SEM (n = 8–14 for control, PMA and MPs; n = 3 for the others). °P < 0.05 versus monocytes; *P < 0.05 versus PMA; ***P < 0.001 versus control. In C: % inhibition of PMA- or MPs-evoked O_2^- production by 15d-PGJ$_2$ (15d-PGJ; 10 µM) in both cell types. Cells were treated with the endogenous PPARγ agonist for 30 min and then challenged with MPs (10 µg·mL^{-1}) or PMA (1 µM). Data are expressed as mean ± SEM of five separate experiments in each cell type. **P < 0.01 versus PMA.

Monocyte-derived MPs induce NF-κB activation in human monocytes and MDM

In unstimulated monocytes, DNA binding of NF-κB is minimal, although detectable, whereas it is greatly increased following challenge with PMA or MPs (Figure 5A). The specificity of the NF-κB DNA binding is confirmed by the reversal of the binding by a 100-fold molar excess of unlabelled probe (data not shown). Nuclear translocation of the transcription factor is maximal when monocytes are stimulated by PMA

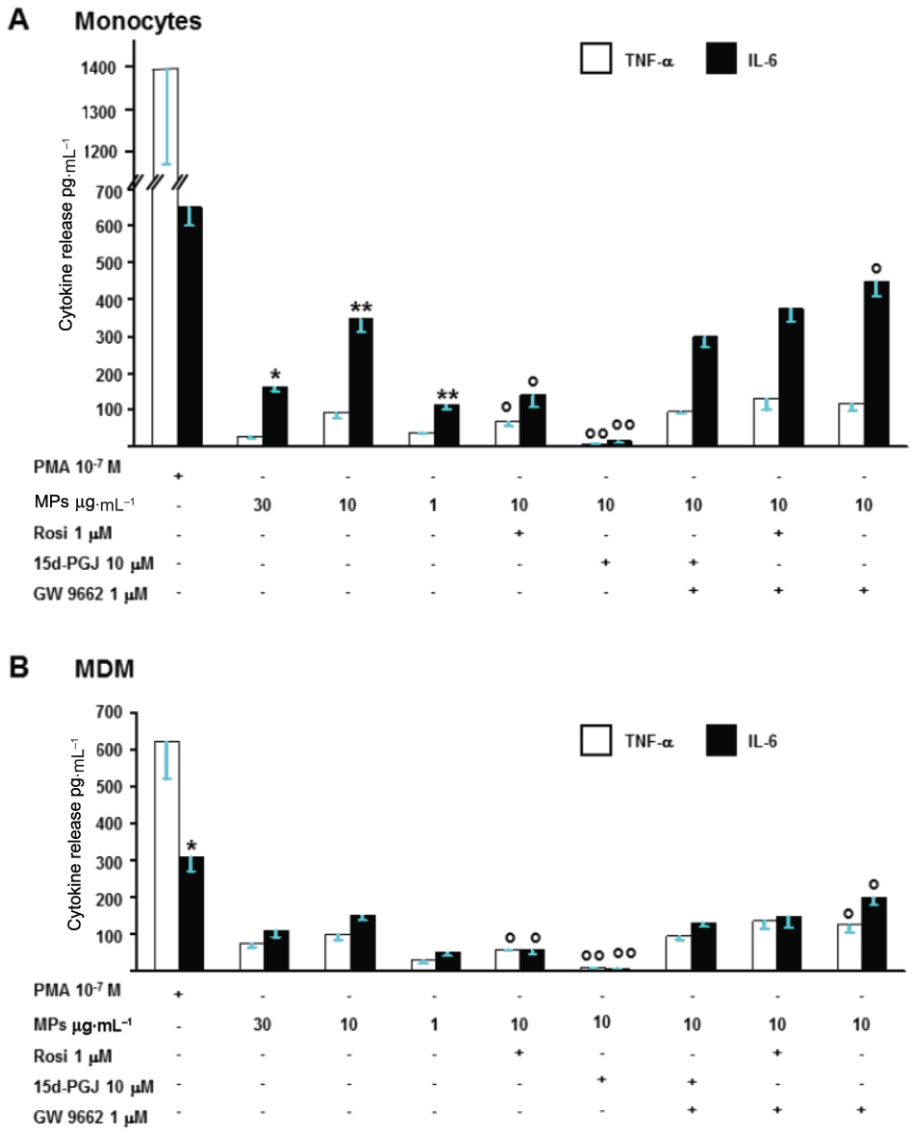

Figure 4

Monocyte-derived MPs induce cytokine production in monocytes (A) and MDM (B). Concentration-dependent (1–30 µg·mL^{-1}) effects of MPs and modulation by PPARγ agonists (15d-PGJ$_2$ (15d-PGJ; 10 µM), rosiglitazone (Rosi, 1 µM) and antagonist (GW9662, 1 µM). TNF-α and IL-6 release by PMA 10^{-7} M are shown for comparison. Values are mean ± SEM; $n = 5$. *$P < 0.05$ versus TNF-α; **$P < 0.01$ versus TNF-α; °°$P < 0.01$ versus MPs 10 µg·mL^{-1}; °$P < 0.05$ versus MPs 10 µg·mL^{-1}.

(1 µM) for 1 h (>threefold increase vs. control; Figure 5A), or by TNF-α 10 ng·mL^{-1} (data not shown). MPs, evaluated at different concentrations (1, 5, 10 and 30 µg protein mL^{-1}), also enhance NF-κB nuclear migration, with maximal effect at 10 µg·mL^{-1} (Figure 5A). Previous reports (Tesse *et al.*, 2008; Amoruso *et al.*, 2009b, 2010; Neri *et al.*, 2011) documented the ability of PPARγ agonists to inhibit NF-κB activation. When used at a near maximal concentration (5 µM), rosiglitazone potently inhibited MP-triggered NF-κB translocation, yielding values similar to unstimulated monocytes in densitometric evaluations, while it was only modestly effective in reducing PMA-induced translocation (Figure 5A).

In human MDM, MPs exert a concentration (1–30 µg protein mL^{-1})-dependent effect and, at the highest 10 and 30 µg·mL^{-1} concentrations, were even more potent than PMA in evoking NF-κB nuclear migration (Figure 5B). EMSA quantification of NF-κB translocation (setting unstimulated MDM = 1) is illustrated in the lower half of Figure 5B; the effect of 10 µg·mL^{-1} MPs was almost double that of PMA (Figure 5B). In human MDM, rosiglitazone potently reduced NF-κB activation evoked by MPs, while it was less effective against PMA-induced activation (Figure 5B).

In order to confirm that MPs, rather than soluble molecules present within supernatant, were responsible for NF-κB nuclear translocation, we performed some experiments with ultra-centrifuged (100 000× *g*) materials, as well as direct stimulation with the calcium ionophore. As shown in Figure 6, in human monocytes, NF-κB translocation induced

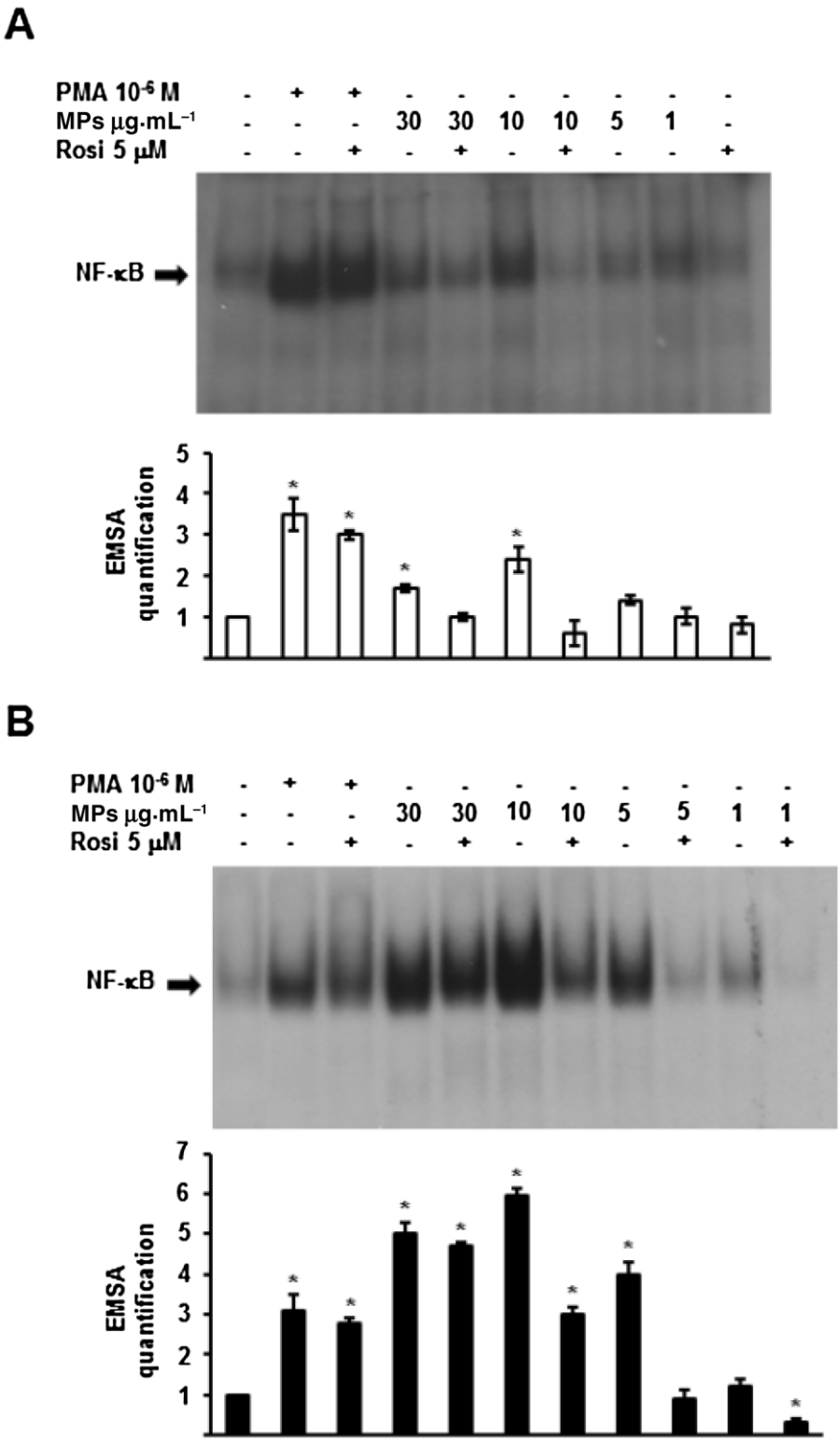

Figure 5

Monocyte-derived MPs induce NF-κB activation in human monocytes (A) and MDM (B). Concentration-dependent (1–30 µg·mL^{-1}) effects of MPs and modulation by rosiglitazone (Rosi, 5 µM) and a PPARγ antagonist (GW9662, 1 µM). The effects produced by PMA 1 µM are shown for comparison. Histograms below each blot are densitometric analysis for EMSA quantification, control, unstimulated cells being = 1. Mean ± SEM; $n = 4$. *$P < 0.05$ versus control.

Table 1

Rosiglitazone (1 μM) and 15d-PGJ$_2$ (10 μM) inhibit PMA- and MPs-evoked cytokine release in all cell types

	% inhibition of MPs 10 μg·mL^{-1}		% inhibition of PMA 10^{-7} M	
	TNF-α	IL-6	TNF-α	IL-6
Monocytes (n = 5)				
Rosi	27 ± 1	60 ± 6**	62 ± 2§§	58 ± 3
15d-PGJ$_2$	92 ± 1°°	96 ± 1°°	95 ± 2°°	82 ± 4°°
MDM (n = 5)				
Rosi	28 ± 5	30 ± 3	40 ± 8	42 ± 7
15d-PGJ$_2$	40 ± 6	54 ± 5°°	52 ± 3	58 ± 5

Cells were pretreated with (or without) PPARγ agonists for 1 h and then stimulated by PMA or MPs for 24 h; the percentage of cytokine release inhibition produced by each agonist was calculated. Rosi: rosiglitazone. Data are expressed as mean + SEM of five experiments.
**P < 0.01 versus TNF-α; °°P < 0.01 versus rosiglitazone; $^{§§}P$ < 0.01 versus MPs.

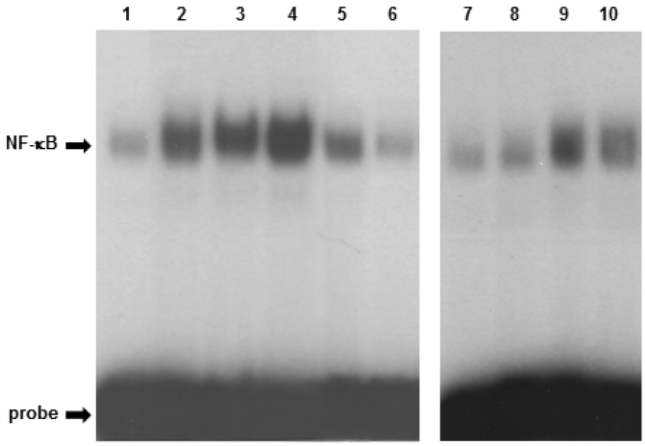

Figure 6

Monocyte-derived MPs, but not ultra-centrifuged supernatants, induce NF-κB activation in human monocytes. Lane 1: un-stimulated monocytes; lane 2: +PMA 10^{-6} M; lane 3: +MPs 30 μg·mL^{-1}; lane 4: +MPs 10 μg·mL^{-1}; lane 5: +ultra-centrifuged pellet (300 μL) from A 23187-stimulated monocytes; lane 6: +ultra-centrifuged supernatant (300 μL) from A23187-stimulated monocytes; lane 7: unstimulated monocytes; lane 8: +supernatant (300 μL) from un-stimulated monocytes; lane 9: +A23187 1.2 μM; lane 10: +A23187 4 μM. This blot is representative of another one.

by MPs (10 or 30 μg protein mL^{-1}) is significantly higher than that evoked by A23187 (used at 1.2 and 4 μM, corresponding to 100 and 300 μL aliquots of supernatant). As expected, supernatants from both un-stimulated monocytes and ultra-centrifuged materials were devoid of activity. The pellet from the ultra-centrifuged samples still induced some NF-κB translocation, but it was less effective (Figure 6), as already observed for superoxide anion production (see Figure 3B). To explain this apparent reduced activity, we suggest that a loss of bioactive material occurs during the experimental procedures or, alternatively, there is a higher 'concentrated' MPs content. By evaluating total protein concentration, we have observed that 300 μL of ultra-centrifuged pellet contain about 40 μg protein (instead of 30 μg protein, as usually observed in MPs). As the activity of MPs follows a bell-shaped dose-response curve, the 30 μg·mL^{-1} concentration displaying a lesser effect than the 10 μg·mL^{-1} concentration, it is conceivable that a more elevated concentration (as in the case of ultra-centrifuged pellet) could exert a lower activity.

Table 2

PPARγ protein expression in human monocytes and macrophages

Treatment	Monocytes (n = 5)	MDM (n = 5)
Control	0.4 ± 0.03	1.5 ± 0.1°°
+15d-PGJ$_2$ (10 μM)	0.9 ± 0.05*	3 ± 0.2*
+Rosi (1 μM)	1.1 ± 0.1*	3.5 ± 0.2*
+MPs (10 μg·mL^{-1})	0.8 ± 0.1*	2.9 ± 0.2*
+MPs (1 μg·mL^{-1})	0.45 ± 0.1	1.7 ± 0.1

PPARγ protein expression was semi-quantified by measuring the ratio between PPARγ expression and β-actin expression. Rosi: rosiglitazone. Data are expressed as mean ± SEM of five experiments.
*P < 0.05 versus Control (unstimulated cells of the same type); °°P < 0.01 versus control monocytes.

Monocyte-derived MPs induce PPARγ protein expression in human monocytes and MDM

As shown in Table 2, human monocytes constitutively express low, although detectable, levels of PPARγ protein, that are up-regulated during differentiation to MDM, as previously reported (Amoruso *et al.*, 2007, 2009a). The two PPARγ agonists enhance PPARγ protein expression about twofold in both cell types, which is in good accord with previous reports (Amoruso *et al.*, 2007, 2009a). Interestingly, when evaluated at 10 μg protein mL^{-1}, MPs induce PPARγ protein expression in both monocytes and MDM, slightly less than that after the selective ligands (Table 2). A representative Western blot is also provided (Figure 7).

Discussion and conclusions

The results presented in this paper show, for the first time, that MPs generated from A23187-stimulated human monocytes significantly affect human cells of the same lineage. We showed that monocyte-derived MPs induce oxygen radical production, cytokine release, NF-κB activation and PPARγ protein expression in both isolated human circulating monocytes and human macrophages (MDM).

Several studies have demonstrated that MPs from different cell types exert pro-inflammatory activities in monocyte/macrophages (Barry *et al.*, 1998; Distler *et al.*,

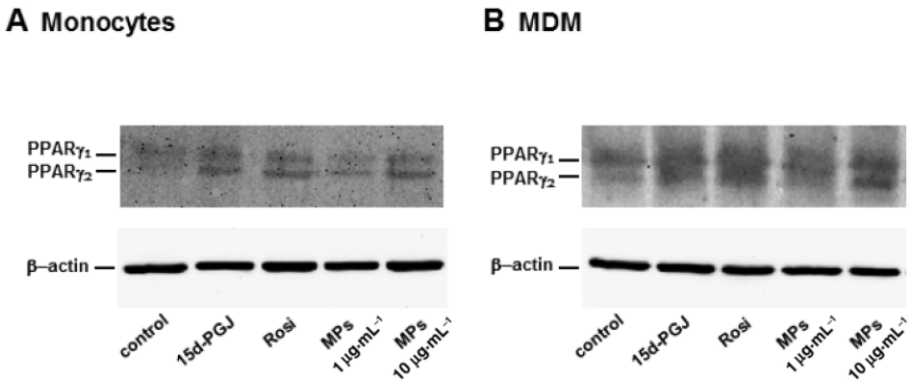

Figure 7

Monocyte-derived MPs induce PPARγ expression in human monocytes (A) and MDM (B). Cells were incubated with 15d-PGJ_2 (15d-PGJ; 10 μM), rosiglitazone (Rosi, 1 μM) or MPs (1 or 10 μg·mL^{-1}) for 6 h. This representative blot shows PPARγ (both one and two isoforms) and β-actin expression. The corresponding quantification is presented in Table 2.

2005a; Baj-Krzyworzeka *et al.*, 2007; Scanu *et al.*, 2008; Carpintero *et al.*, 2010), but our novel finding is that these effects are produced even by monocyte-derived MPs. These results suggest that MPs may not only mediate cell-to-cell communication but may also represent a key component for autocrine stimulation.

Berckmans *et al.*, (2005) have demonstrated that synovial fluid MPs induced an enhanced release of CCL2, IL-8 and IL-6 in fibroblast-like synoviocytes. However, as stated by the authors (Berckmans *et al.*, 2005), synovial fluid MPs are mainly derived from monocytes and neutrophils, and therefore may not represent an autocrine effect.

There are two well-known processes which lead to MPs formation: (i) chemical and physical cell activation (e.g. activated complement components, PMA, bacterial LPS, calcium ionophores, inflammatory cytokines, shear stress) and (ii) apoptosis, induced by growth factor deprivation or pro-apoptotic agents. As far as human monocytes are concerned, Essayagh *et al.* (2007) produced MPs from apoptotic monocytes, challenged with the Fas ligand (0.2 μg·mL^{-1} for 6 h), whereas Gauley and Pisetsky (2010) used LPS (0.05–50 μg·mL^{-1}) for 24 h to induce MPs formation in RAW 264.7 cells, and stated that, under these conditions, MPs production resulted either from cells undergoing apoptosis or from activated cells. Moreover, Aharon *et al.* (2008) used starvation (20 h) or challenge with LPS (1 μg·mL^{-1}) for 5 h + A23187 (10 μM) for 15 min to induce MPs generation in THP-1 cells. We prepared MPs as previously described (Neri *et al.*, 2011), by treating monocytes with A23187 (12 μM). We are aware that calcium ionophores are not physiological activators, but A23187 induces a rapid and complete MPs release, and is regularly used experimentally. For example, Satta *et al.* (1994), although demonstrating MPs formation from human monocytes challenged with LPS (5 μg·mL^{-1} for 5 h), used A23187 (3 μM) to characterize monocyte and shed MP populations. These authors also reported that, using LPS, the degree of MPs formation 'was strongly donor-dependent'. In addition, Cerri *et al.* (2006) demonstrated that A23187 (1–24 μM) evokes a concentration-dependent MPs release from human monocytes, 12 μM representing the EC_{50} concentration. According to these authors, histamine, at 30 μM, induces MPs formation from human monocytes, but it requires a longer stimulation time (1–2 h) instead of 10 min (Cerri *et al.*, 2006).

Characterization of MPs is neither a standardized nor unequivocal procedure: we have addressed this problem by evaluating the percentage of $CD14^+$ elements, TF activity, PS equivalent concentration, as well as protein concentration in the samples. In our MPs preparations, 100 μL of supernatants from A23187-challenged monocytes corresponds to 10 μg protein (that, in our experiments, usually ensures maximal effects) and was 2 nM in PS equivalents, in good agreement with other reports (Scanu *et al.*, 2008; Tesse *et al.*, 2008; Carpintero *et al.*, 2010). In fact, Scanu *et al.* (2008) and Carpintero *et al.* (2010) report maximal effects with 30 and 6 μg·mL^{-1} protein concentrations for T cell-derived MPs respectively, whilst Tesse *et al.* (2008), in experiments on vascular reactivity, use 30 nM PS equivalents of T cell-derived MPs.

We can also speculate that monocyte-derived MPs directly interact with monocyte/macrophages, leading to further cell activation, which may represent an amplification loop that perpetuates inflammation. Such a direct interaction is plausible since our MPs preparations are PS-positive and macrophages express PS receptors (Fadok *et al.*, 2000).

Another interesting finding of our study is that monocyte-derived MPs exert concentration-dependent effects, with a somewhat varied sensitivity according to the cell type and/or the type of test. As previously documented (Essayagh *et al.*, 2007), MPs generated from apoptotic monocytes induce the production of reactive oxygen species (ROS), mainly superoxide anion, by human endothelial cells, as well as transient platelet recruitment and TF expression. Our results substantiate and further extend this observation, since we demonstrate that in human monocytes and MDM (which are major phagocytes and present a functionally active NADPH oxidase), monocyte-derived MPs induce a concentration-dependent O_2^- production, that peaks at 10–30 μg·mL^{-1} protein concentration.

Furthermore, in keeping with previous studies in other cell types (Mesri and Altieri, 1999; Tesse *et al.*, 2008; Neri *et al.*, 2011), this study provides evidence that monocyte-

derived MPs induced, in a concentration-dependent manner, TNF-α and IL-6 release from human monocyte/macrophages, and also demonstrated that rosiglitazone and 15d-PGJ_2 reduced these effects. Amoruso *et al*. (2008, 2009b, 2010) have shown that, at maximally effective concentrations, 15d-PGJ_2 often exerts a significantly higher inhibition than rosiglitazone, which is independent of the stimulus (except in the case of TNF-α release in human monocytes). Tesse *et al*. (2008) previously documented that rosiglitazone, used at 5 μM, prevented the increase of IL-6, IL-1β and IL-8 mRNA expression induced by T cell-derived MPs in human endothelial cells. In human lung epithelial cells, Neri *et al*. (2011) demonstrated that monocyte-derived MPs increased IL-8 and CCL2 synthesis, which was inhibited by rosiglitazone and 15d-PGJ_2. This inhibitory effect is likely to be mediated via interaction with PPARγ as rosiglitazone and 15d-PGJ_2 are PPARγ agonists. However, since both rosiglitazone and 15d-PGJ_2 have the potential to exert biological effects independent of PPARγ activation, we used the specific PPARγ inhibitor GW9662 to evaluate the role of this receptor in the modulation of MPs-induced cytokine release. As the antagonist completely reversed the effects of the two PPARγ agonists, we confirmed the direct involvement of this receptor. Moreover, another PPARγ agonist, pioglitazone, has been shown to reduce the circulating levels of endothelial MPs in the metabolic syndrome (Esposito *et al*., 2006), with an effect independent of its ability to ameliorate insulin sensitivity.

Some of the anti-inflammatory properties of PPARγ are attributed, at least partially, to its *trans*-repression ability. PPARγ can physically interact with the p65 subunit of NF-κB, so preventing its nuclear translocation (Chen *et al*., 2003; Tesse *et al*., 2008), or it can be sumoylated and indirectly inhibit NF-κB binding (Pascual *et al*., 2005). In our hands, monocyte-derived MPs induced NF-κB nuclear translocation, which was significantly reduced by PPARγ agonists, in good agreement with previous reports (Tesse *et al*., 2008; Neri *et al*., 2011). Moreover, MPs-induced NF-κB nuclear migration was similar to that evoked by PMA and significantly higher than that induced by direct A23187 stimulation.

Another novel finding of our research is that monocyte-derived MPs stimulated (about twofold) PPARγ protein expression in human monocytes and MDM, with maximal effects that were slightly lower than those induced by the selective ligands rosiglitazone and 15d-PGJ_2. Therefore, these results support and further extend previous data describing the anti-inflammatory properties of MPs (Gasser and Schifferli, 2004; Koeppler *et al*., 2006; Dalli *et al*., 2008). For example, the transfer of Kato cell-derived MPs to human monocytes results in a decreased release of the pro-inflammatory cytokines GM-CSF and TNF-α and an enhanced release of the anti-inflammatory IL-10 (Koeppler *et al*., 2006). The release of PPARγ, in association with platelet MPs, is another intriguing finding in the literature (Ray *et al*., 2008). Platelet MPs can be internalized by the monocytic THP-1 cells and, in their presence, rosiglitazone significantly attenuates THP-1 activation, suggesting a novel transcellular mechanism of regulation (Ray *et al*., 2008).

We now demonstrate that monocyte-derived MPs not only induce pro-inflammatory effects (ROS production, cytokine release, NF-κB activation) in human monocyte/macrophages, but also enhance PPARγ protein expression in the same cells, so providing a possible counter-regulatory, anti-inflammatory mechanism. PPARγ is highly expressed in human MDM and alveolar macrophages (from healthy individuals or animal models), whereas the amount of PPARγ protein in monocytes is markedly lower (Asada *et al*., 2004; Reddy *et al*., 2004; Amoruso *et al*., 2007, 2008).

In conclusion, these results demonstrate that monocyte-derived MPs have relevant pro-inflammatory effects, that are reduced by PPARγ agonists, in human monocytes and MDM. This observation suggests that monocyte-derived MPs are relevant to autocrine stimulation in human cells. Moreover, our results also indicate a novel activity, enhancement of PPARγ protein expression, for monocyte-derived MPs, that might support a possible anti-inflammatory loop.

Since MPs represent a disseminated storage pool of bioactive effectors, their properties need to be carefully evaluated, according to the nature of MPs and particular recipient cells, given the possibility of dual effects of MPs.

Acknowledgements

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Conflict of interest

None.

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α2M-microcapsules

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

Federici Canova D, Norling LV, Pavlov A, Sukhorukov GB, Brunelleschi S, Perretti M

During the third year of my PhD program in Prof. Perretti's lab (Queen Mary University of London), I became interested in microcapsules, especially those enriched with alpha-2-macroglobulin (α2M). For this project, it has been supposed to exploit the features of MPs, mainly derived from phagocytes, as endogenous controllers of the inflammatory process and to recapitulate their biological function in synthetic structures [104]. Indeed, a new technology is currently available that allows the construction of microcapsules with defined size, composition and content of encapsulated materials [67]. Capsules are synthesized 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 these new capsules enriched with key compounds of inflammatory process, in order to mimic the effects of the natural MPs on the process of interest and on specific target cells, in absence of all the others mediators present in endogenous MPs. This can represent an advantageous tool to better understand the biological function of specific inflammatory mediators.

Among the several mediators of the inflammatory process discovered in MPs, α2M is of particular interest. Actually, in a previous work in Perretti's lab, α2M has been found abundant in a specific subset of neutrophil-derived MPs that demonstrate protective role in sepsis, characterized in some cases by an immune paralysis and neutrophil dysfunction (submitted) (see Introduction).

The aim of this study was to generate and evaluate the biological property of α2M-microcapsules in *in vitro* assays of neutrophil-endothelial cell interaction (flow chamber) and in phagocytosis assay using human monocyte-derived macrophages (MDM), isolated from peripheral blood of healthy volunteers. We wanted to verify if this capsules technology could actually mimic the effects of the endogenous MPs in the absence of the other proteins. Furthermore, this could represent an important step in manufacturing microcapsules enriched with biologically relevant proteins hence with potential therapeutic/delivery application.

First, microcapsules were generated and characterized. Blank and α2M-microcapsules (see methods section) 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. 9A) and flow cytometry (Fig. 9B) in comparison with 1μm fluorescent beads: their size results to be approximately between 1-4 μm, as the endogenous MPs are. Moreover, microcapsules were checked for their α2M content by Western Blot using a specific anti- α2M antibody (Fig. 9C), confirming the expected characteristics.

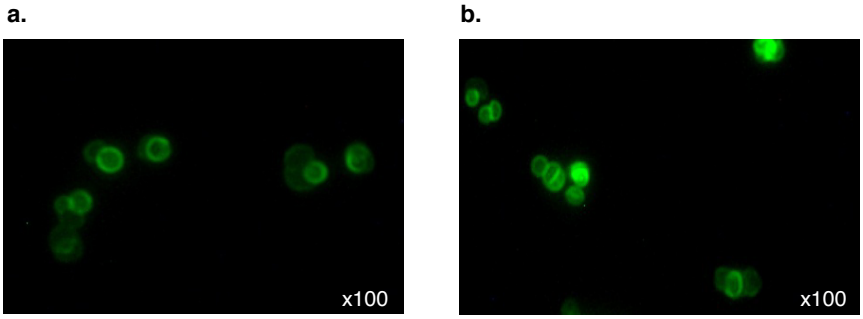


Figure 9A. ***Fluorescent microscope analysis of microcapsules.*** α2M-microcapsules (a.) and blank-microcapsules (b.) were visualized under fluorescent microscope thanks to their conjugation with FITC probe.

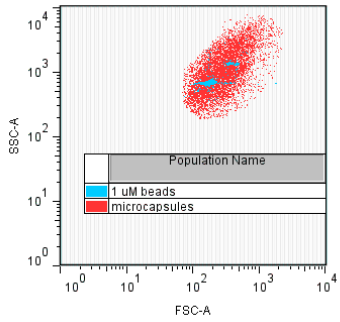


Figure 9B. ***FACS analysis of microcapsules.*** α2M-microcapsules (red dots) were controlled by flow cytometry using the forward and side scatter parameters, together with 1μm beads for comparison (blue dots).

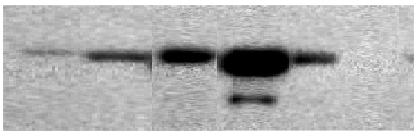


Figure 9C. ***Western blot analysis of microcapsules.*** α2M-microcapsules and blank-microcapsules were loaded into WB gel and incubated with anti-α2m antibody. Lane 1-4: free α2m 0.1-0.5-1-5 μg; lane 5: α2M-microcapsules; lane 6: blank-microcapsules.

α2M-microcapsules and blank microcapsules were then 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. 10A). Of note, α2M-microcapsules had similar effects of soluble active α2M (10nM) (Fig. 10B).

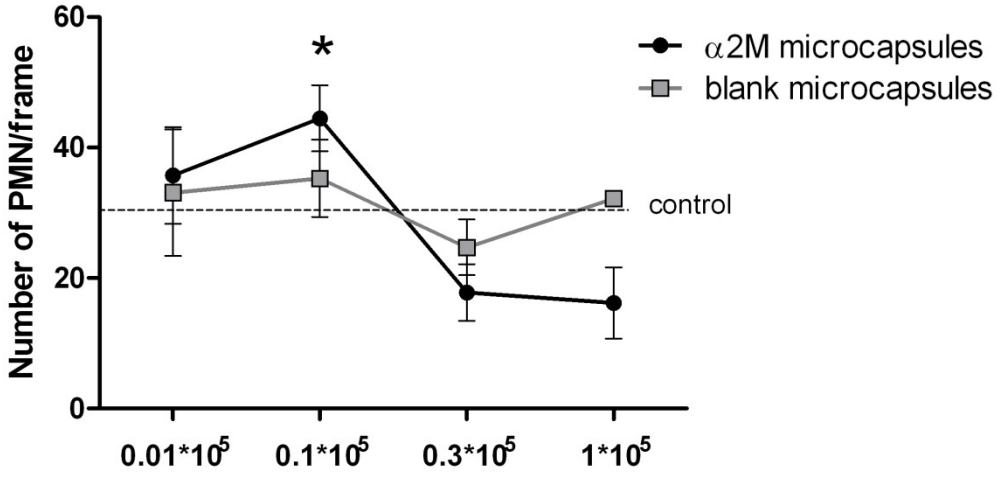


Figure 10A. ***Flow chamber assay.*** Endothelial cells were incubated with TNFα (10ng/ml) in presence or absence of α2M-microcapsules or blank microcapsules for 4h, at 37°. Then, freshly prepared neutrophils were perfused over the monolayer and the number of adherent neutrophils was quantified. The 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 $0.1*10^5$).

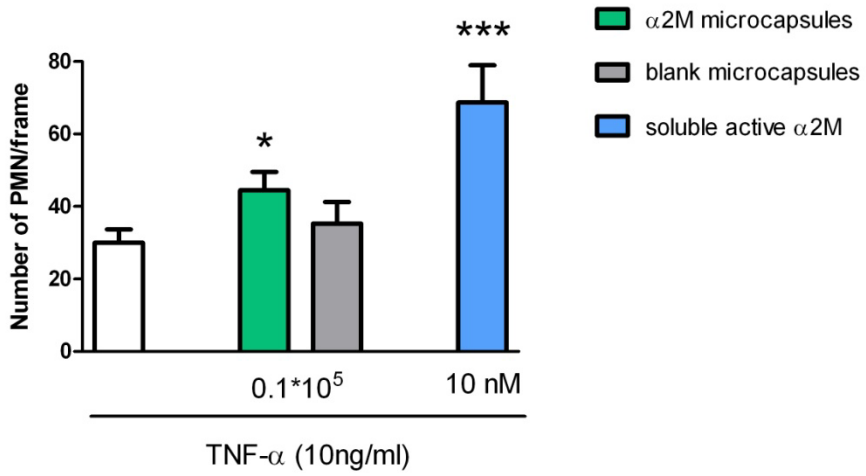
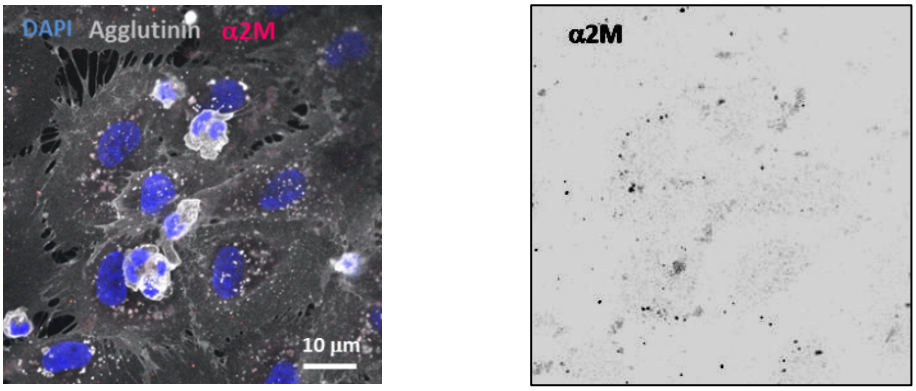


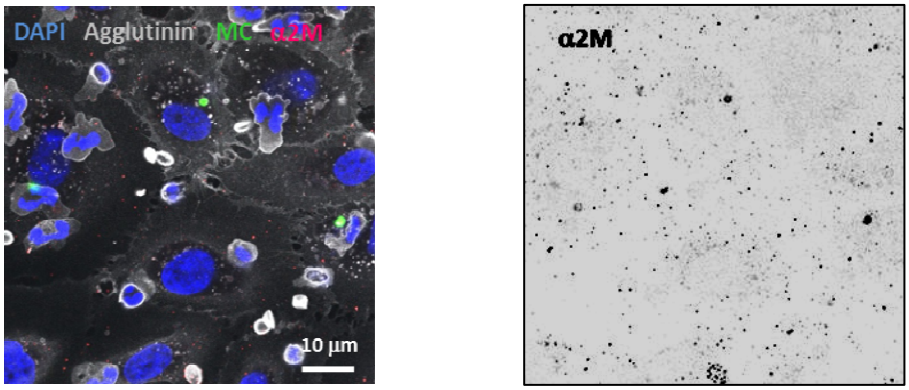
Figure 10B. ***Flow chamber assay****.* The effectsof microcapsules were compared to those of soluble active α2M used at 10nM. Endothelial cells were incubated with TNFα (10ng/ml) in presence or absence of α2M-microcapsules, blank microcapsules or soluble α2Mfor 4h, at 37°. Data are mean ± SEM of 4-12 differ ent HUVEC and neutrophils preparations (Unpaired t-test: *p<0.05 *vs.* control).

After the flow experiments, monolayer 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. 11A). Confocal microscopy images demonstrate how incubation with α2M-microcapsules significantly increases the expression of α2M on the surface of endothelial cells that is not restricted to the area where the α2M-microcapsules were present. This effect was not replicate in the case of cell incubation with blank-microcapsules (Fig. 11B).

a. TNF-α stimulated endothelial cells



b. α2M-microcapsules stimulated endothelial cells



c. blank-microcapsules stimulated endothelial cells

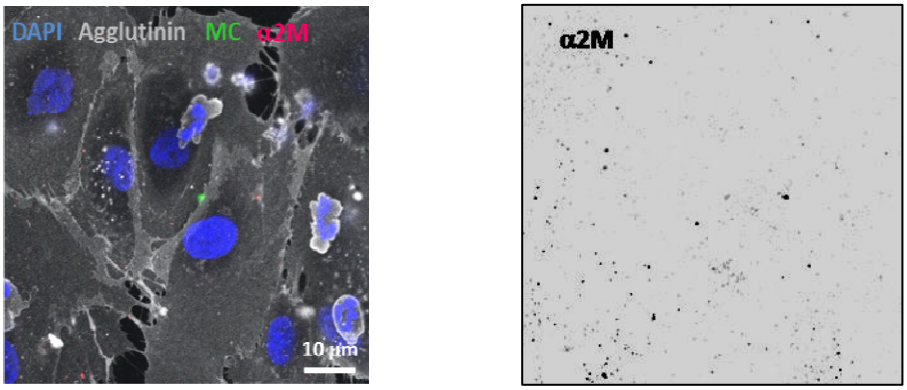


Figure 11A. ***Confocal images after flow experiments***. After the flow chamber assay, cells were labelled with fluorescent Agglutinin (grey staining) and an anti-α2M antibody (red staining). On the right panel of each treatment (**a**. control; **b**. α2M-microcapsules; **c.** blank-microcapsules) 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.

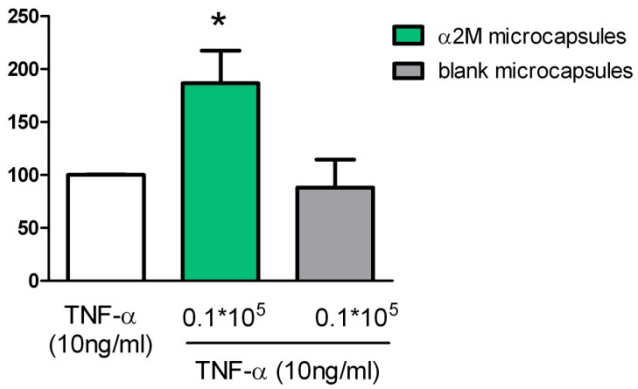


Figure 11B. ***Confocal images after flow experiments***. After the flow chamber assay, cells were labelled with anti-α2M antibody (1h at 4˚ in the dark) and the number of positive particles was acquired using ImageJ software and expressed as percentage over control. Data are mean ± SEM of 3 different flow chamber experiments (Unpaired t-test: *p<0.05 *vs.* control).

α2M-microcapsules 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 MPs 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 LRP-1 (cluster definition CD91) is generally recognized as a macrophage receptor critical for the engulfment of apoptotic cells [76]. Therefore, we initially tested if LRP-1 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. 12A) and CD68 for macrophages (Fig. 12B). Cell preparation was run untouched or following permeabilization with saponin, thus quantifying both cell surface and intracellular receptor expression (Fig. 12A, 12B). 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 LRP-1, they may express it on the surface just upon activation.

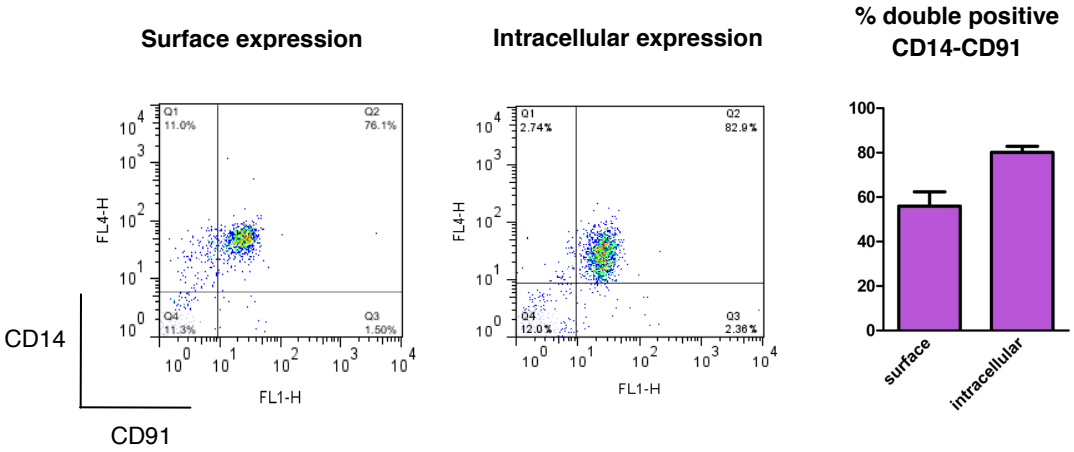


Figure 12A. ***FACS analysis of human monocytes.*** Cells were labeled with fluorescently conjugated anti-CD14 and anti-CD91 antibodies for 1h at 4˚ in the dark. Data are mean ± SEM of 4 different monocytes preparations.

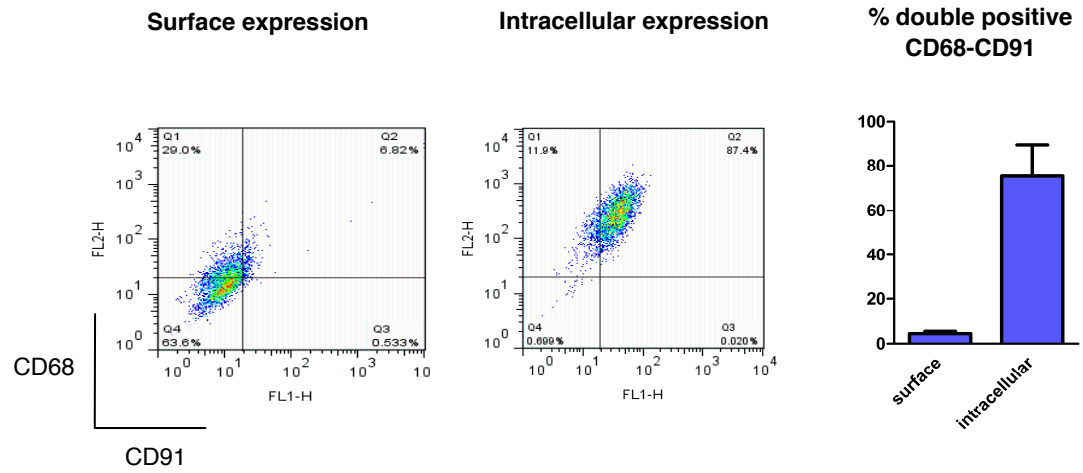


Figure 12B. ***FACS analysis of human MDM.*** Cells were labeled with fluorescently conjugated anti-CD68 and anti-CD91 antibodies for 1h at 4˚ in the dark. Data are mean ± SEM of 4 different macrophages preparations.

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. 13A) were added to cells and phagocytosis was monitored. As depicted in Fig. 13B, incubation of MDM with

α2M-microcapsules can significantly promote their ability to phagocyte Zymosan particles, in a concentration dependent manner, with more efficiency compared to blank-microcapsules, especially at the higher amount of microcapsules. Moreover, these effects were similar to those of soluble active α2M (1nM and 10nM).

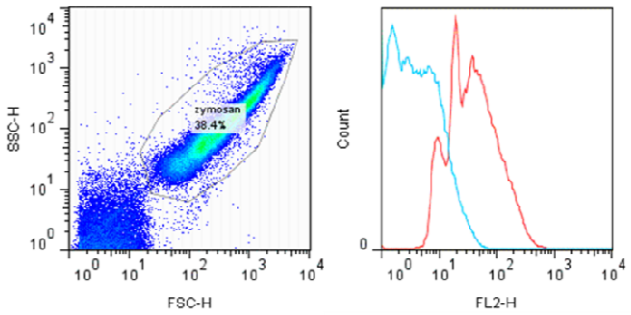


Figure 13A. ***FACS analysis of conjugated Zymosan particles***. The particles of Zymosan were conjugated with a fluorescent dye (Bodipy, 1μM) and then verified by FACS (blue line: PBS; red line: conjugated Zymosan).

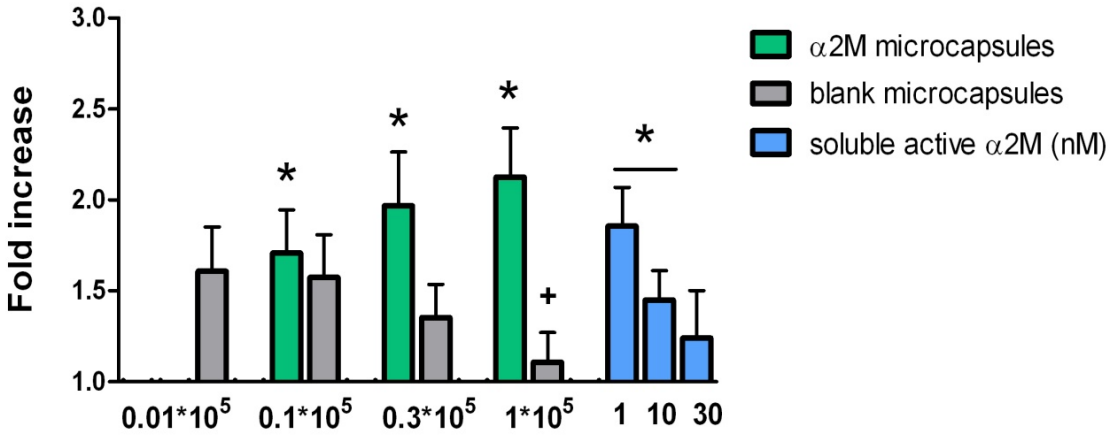


Figure 13B. ***Stimulation of MDM with α2M-microcapsules increases their ability to phagocyte Zymosan particles***. MDM were incubated with α2M-microcapsules, blank microcapsules or soluble active α2M for 24h (at 37°). The particles of Zymosan were added to cells at a final concentration of 125 μg/ml,for 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, **p<0.01 *vs.* control, +p<0.05 *vs.* α2M-microcapsules same amount).

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 occurs in sepsis. Therefore, MDM were first incubated with microcapsules and soluble active α2M for 24h and then

fluorescent conjugated *E.Coli* particles (Fig. 14A) were added to cells and phagocytosis was monitored. As depicted in Fig. 14B, again, incubation of MDM with α2M-microcapsules significantly enhanced their ability to phagocyte bacterial particles, with more efficiency compared to blank-microcapsules, even if they seem to have some unspecific effects. However, at the higher amount, α2M-microcapsules display significant different effects on phagocytosis compare to blank-microcapsules. Similarly to Zymosan phagocytosis, the effects of α2M-microcapsules were comparable to those of soluble active α2M (10 nM) also on *E. Coli* engulfment.

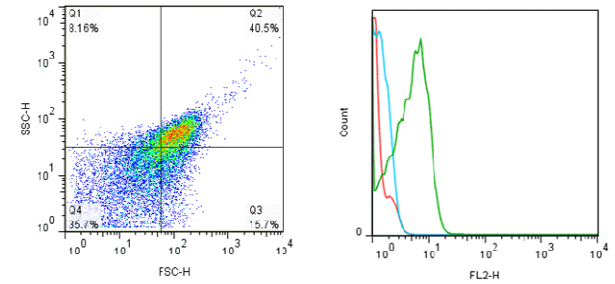


Figure 14A. ***FACS analysis of conjugated E. Coli particles***. The particles of *E. Coli* were conjugated with a fluorescent dye (Bodipy, 1μM) and then verified by FACS (blue line: PBS; red line: un-conjugated *E. Coli*; green line conjugated *E. Coli*).

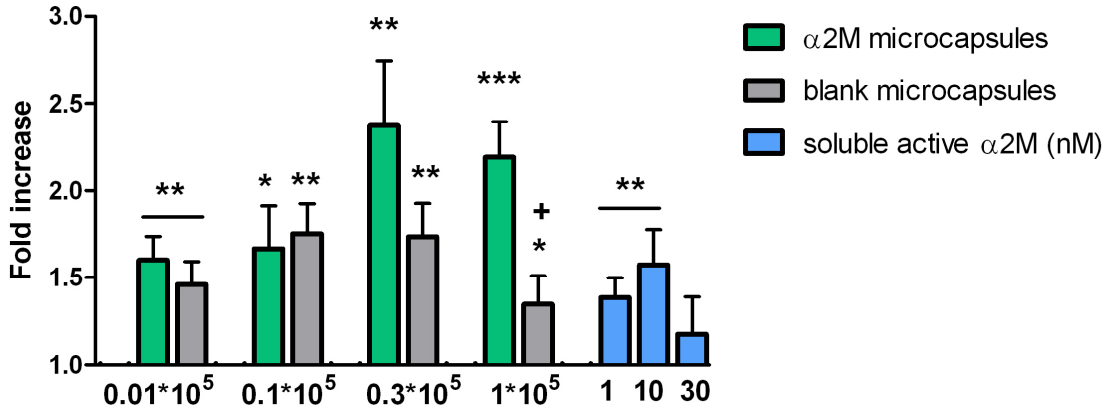


Figure 14B. ***Stimulation of MDM with α2M-microcapsules increases their ability to phagocyte E. Coli particles***. MDM were incubated with α2M-microcapsules, blank microcapsules or soluble active α2M for 24h (at 37°). The particles of *E. Coli* were added to cells at a final concentration of 1mg/ml,for further 60 min. The number of phagocytosed particles was determined with a fluorescence plate reader. Data (fold increase over basal) are mean ± SEM of 4 different experiments (Unpaired t-test: *p<0.05, **p<0.01, ***p<0.001 *vs.* control, + p<0.05 *vs.* α2M-microcapsules same amount).

In conclusion, in these experiments we observed that α2M-microcapsules mimicked the actions of neutrophil derived MPs and soluble α2M, displaying significant effects on both human neutrophils and 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 we moved to assess biological properties of these microcapsules on macrophages. This cell type has been often studied in the context of MPs biology. For instance, atherosclerosis plaques contain MPs that mainly derive from monocyte/macrophages and there are also evidences that monocyte-derived MPs can contribute to the inflammatory process and to the destruction of cartilage and bone in rheumatoid arthritis (RA) [54]. In addition, as recently demonstrated by our group, monocyte-derived MPs can induce the expression of PPARγ in monocytes and macrophages, thus switching the status of cell activation upon application of specific receptor ligands [105]. Finally, macrophages respond to α2M [76] and express LRP-1 [89]. It was therefore important to test the α2M-microcapsules on this cell type. According to our results, only α2M-microcapsules, but not blank-microcapsules, increased 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) [106]. 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. We have generated proof-of-concept data showing how the microcapsule technology can be used to exploit biological functions of bioactive proteins, especially those that we have identified in human MPs (α2M, but also Annexin A1, Lactoferrin and Cerulosplasmin).

DISCUSSION and CONCLUSIONS

Inflammation is a protective and complex reaction that the organism orchestrates against injuries, infection or tissue damage in general. Despite a lot of efforts in medical research, it is still necessary to find and develop novel anti-inflammatory medications and new therapeutic targets to treat inflammatory diseases, especially the chronic ones, as rheumatoid arthritis or atherosclerosis.

Recently, new chemical strategies to modulate inflammation are emerged. Indeed, it has been thought to take advantage of the anti-inflammatory potential of nitric oxide (NO), which, in physiological condition, has anti-thrombotic effects, promoting vasodilatation, inhibiting platelets aggregation and smooth muscular cells migration and proliferation and, in the immune system, it inhibits leucocytes adhesion and the release of inflammatory mediators [8,12,13]. A new class of drugs that combine the property of a slow release of NO with the inhibition of cholesterol synthesis have been recently developed and called nitro-statins [107]. During my PhD I was involved in the study of the effects of NO-conjugated pravastatin (NCX-6550) on monocytes and macrophages, in comparison to native drug pravastatin, focusing on key inflammatory pathways. Previous studies had reported that NCX-6550 has higher lipophilicity and an increased anti-proliferative and anti-inflammatory activity compared to pravastatin [23,24]. Our results further confirmed its anti-inflammatory properties, since we showed that NCX-6550 inhibits superoxide anion production, pro-inflammatory cytokines release and NF-κB activation, while enhancing PPAR-γ gene and protein expression. Our data finally indicate that the incorporation of NO moiety in the structure of pravastatin could result in beneficial effects for the long-term treatment of chronic inflammatory diseases, such as atherosclerosis. However, it has to be noted that, despite years of experimental research, neither nitro-statins nor other NO-conjugated drugs have been marketed and used at clinical level. The most recent case regards naproxen, one of the most common non-steroidal anti-inflammatory drugs, and its related nitro-naproxen or Naproxcinod. Despite this new compound maintains COX-inhibiting properties and has beneficial effects on blood pressure and less gastrointestinal side effects [108,109], the Food and Drug Administration (FDA) had established that still there aren't sufficient evidences regarding its long-term safety. Therefore, it is still required to further investigate and provide indications of the efficacy and safety of these new nitro-drugs, especially for long-term treatments.

In the last few years, more and more studies have tried to elucidate the role of a new protagonist in the scenario of inflammation, the microparticles (MPs). MPs are small membrane-bound vesicles, first described as inert subcellular elements release from several cell types, upon activation or apoptosis [29]. It has now become clear that they actually play a key role in several processes, because they transport different mediators that can be transferred to the recipient cells and finally affect their response. In an unpublished work, MPs have been showed to contain about 400 proteins and to have partial distinct proteome in relation to the stimulus applied for their generation. This can explain, at least in part, the controversial results in literature regarding the discrepancy in their biological functions, even when they are produced from the same cell type, displaying pro- or anti-inflammatory properties. Part of my PhD program dealt with monocyte-derived MPs, which were previously shown to affect inflammatory response, for instance in human fibroblast and airway epithelium [54,56,57]. We have demonstrated that monocyte-derived MPs have both pro-inflammatory and anti-inflammatory effects when added to human monocytes and macrophages. Indeed, they induced superoxide anion production, cytokines release and NF-κB nuclear translocation (all pro-inflammatory activities), but also enhanced the expression of a key anti-inflammatory receptor, PPAR-γ, upon MPs stimulation [105], supporting other studies regarding the anti-inflammatory action of MPs [31,60].

As explained, endogenous MPs contain a wide spectrum of protein. However, it is of considerable importance that, in an unpublished work, Perretti' s group reported that a specific subset of neutrophil-derived MPs was abundant of alpha2-macroglobulin (α2M), which is a plasma protein that recognizes and tightly binds several growth factor and cytokines and almost all the types of proteinases [68-70]. Increased levels of this specific subset of MPs were found in the plasma of patients that survived sepsis compared to non-survivors patients or healthy donors, and α2M was found to exert powerful stimulatory effects on neutrophil responses. Therefore, it was decided to exploit the features of MPs as endogenous controllers of the inflammatory process and to recapitulate their biological function in synthetic structures [104]. Since a new technology is now available that allows the construction of microcapsules with defined size, composition and content of encapsulated materials [67], the project of the last year of the PhD was founded on the idea to use these new capsules enriched with key components of the inflammatory process, in order to mimic the effects of the natural MPs (in absence of all the others mediators present in endogenous MPs).The capsules used in my 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 this disease. We demonstrated 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, enhancing therefore the phagocytosis process.

In conclusion, during my PhD I have evaluated different and novel approaches to modulate the inflammatory process: NO-conjugated statins, monocyte-derived MPs and microcapsules. Regarding the latter strategy, we have shown that microcapsules could be used to exploit biological functions of key modulators of inflammation and it would be desirable to further investigate their features, also with different cargo, in order finally to use them as potential tools for therapeutic/delivery applications.

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