UNIVERSITY OF PIEMONTE ORIENTALE

"A. AVOGADRO"

Dept. Medical Science

INVOLVEMENT OF MONOCYTE-DERIVED MICROPARTICLES IN MONOCYTE/MACROPHAGES INFLAMMATORY RESPONSE

XXV Cycle PhD Program in Molecular Medicine

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INTRODUCTION

Microparticles (MPs) are a heterogeneous population of small membrane-bound vesicles released from different cell types. They were first reported in 1967 in human plasma as platelet derived-small fragments (Wolf P, 1967) and, for a long time, 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 (VanWijk *et al*., 2003). 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 (Distler *et al*., 2005a; Dalli *et al*., 2008). Depending on their origin, MPs differ in size (diameter 0.1-1 μm), protein and lipid composition, and effects.

There are two well-known cellular processes that can lead to the formation of MPs: cell activation and apoptosis (VanWijk *et al*., 2003).

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 (Barry *et al*., 1997; Ardoin *et al*., 2007), whereas monocytes and endothelial cells release MPs after stimulation with various pro-inflammatory agents, such as bacterial LPS, cytokines (TNF-α or IL-1) or chemical compounds such as phorbol myristate acetate (PMA), ionomycin or calcium ionophore A23187 (Satta *et al*., 1994; Combes *et al*., 1999). In general, the release of MPs is time- and calcium-dependent. One of the first sign 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 (as later explained) (Hugel *et al*., 2005).

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, that depends mainly on activation of Rho-associated kinase-1, ROCK-1 (Coleman *et al*., 2001). 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 (Distler *et al*., 2005a).

Several studies have proposed that MPs differ according to whether they are produced by activation or by apoptosis, in term of macromolecular composition, both external and cytoplasmatic (Jimenez *et al*., 2003; Bernimoulin *et al*., 2009). This suggests that MPs formation may not be a

uniform process, with the release of MPs differing qualitatively and quantitatively, depending on parent cell types as well as on cellular process triggering their release.

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, as later explained.
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 (VanWijk *et al*., 2003; Morel *et al*., 2011). 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 (Dalli *et al*., 2008). 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.

MPs are different from other subcellular structures such as apoptotic bodies and exosomes (Ardoin *et al*., 2007). 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) respect to MPs (Ardoin *et al*., 2007, Hristov *et al*., 2004). 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 (Ardoin *et al*., 2007, Gasser *et al*., 2003).

The identification of MPs is, therefore, based on size and surface markers expression, flow cytometry being frequently used to analyze the features of MPs. 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, that is also used to identify and enumerate MPs (Ardoin *et al*., 2007).

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 circulate in the blood and transport biologically active compounds, so participating in both local and long-range signalling, and can also transfer and exchange surface molecules or cytoplasmatic components. 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 whit platelet-derived MPs. On the

contrary, elevated numbers of MPs have been reported in several diseases, including acute coronary syndrome, cancer, sepsis, diabetes, atherosclerosis and arthritis (Roos *et al*., 2010). *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 (Berckmans *et al*., 2001). Additionally, MPs interact with factor Va, VIII and IXa, thereby facilitating assembly of prothrombinase complex and can induce TF in monocytes (Ardoin *et al*., 2007). Several *in vivo* evidences also showed that MPs contribute to coagulation: MPs numbers are elevated in different types of disease involving hyper-coagulation, as acute coronary syndromes, whereas MPs generation is reduced in some bleeding disorders, such as Scott Syndrome (VanWijk *et al*., 2003). The procouagulant potential of MPs is not restricted to platelet-MPs, since also monocyte-, endothelial- and lymphocyte-derived MPs present PS at their surface. As hypercoagulation is one of the characteristic of cardiovascular diseases, and altered numbers and procoagulant behaviour of MPs were reported in these conditions, MPs can play a casual 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 cyclo-oxygenase 2 (COX-2) expression in these cells, thereby inducing vasodilatation, *via* an increased production of prostaglandins (Barry *et al*., 1997, 1998). Moreover, lymphocyte-derived MPs induce endothelial dysfunction by alteration of nitric oxide and prostacyclin pathways (Martin *et al*., 2004).

MPs are shed even from tumour cells and they can stimulate metastasis by promoting *in vivo* angiogenesis. This proangiogenic potential seems to derive from the presence of sphingomyelin and TF at the external surface that can stimulate endothelial cell migration, invasion and tube formation (Freyssinet, 2003).

The implication of MPs in inflammation is well documented too. Platelet-derived MPs can induce adhesion of monocytes to endothelium, since they deliver arachidonic acid to endothelial cells, which results in upregulation of intracellular adhesion molecule-1 (ICAM-1) and prostaglandins production, *via* upregulation 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 (Barry *et al*., 1998). Furthermore, platelet-MPs can induce leukocyte-leukocyte aggregation, thanks to the interaction between P-selectin, expressed on platelet MPs, and its ligand on leukocytes (Forlow *et al*., 2000). In addition to platelet-derived MPs, MPs released from other cell types can exert pro-inflammatory activities. Neutrophil-derived MPs induce the expression of IL-6 and monocytes chemotactic protein-1 (MCP-1) in endothelial cells, even if, under certain circumstances, they exert anti-inflammatory effects (Mersi and Altieri, 1999; Gasser and Schifferli, 2004; Dalli *et al*. 2008).

Monocyte-derived MPs are less investigated, but it has been demonstrated that they are highly procoagulant, primarily due to the presence of TF and PS (Satta *et al*., 1994; Hugel *et al*., 2005),

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 (Bernimoulin *et al*., 2009). 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 procoagulant MPs, that can contribute to the pathological hypercoagulability of smokers (Li *et al*., 2010). 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 leukocyte adhesion and transmigration (Mayr *et al*., 2009; Rautou *et al*., 2011). 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-metalloproteinases (MMPs) and cytokines (IL-6, IL-8) in fibroblasts isolated from patients with RA, *via* NF-κB pathway (Distler *et al*., 2005b). 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 (Kanazawa *et al*., 2003). Moreover, it has been showed that monocyte-derived MPs affect human bronchial epithelial and alveolar cell lines (Cerri *et al*., 2006; Neri *et al*., 2011). 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 (Cerri *et al*., 2006). In addition, Neri and co-authors (2011) demonstrated, in the same cell lines, that monocyte-MPs induced pro-inflammatory response was mediated by NF-κB activation and modulated by Perixosome Proliferation-Activated Receptor-gamma (PPARγ) (Neri *et al*., 2011). PPARγ 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 important role in the regulation of inflammation. Indeed, activation of PPARγ 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 (Amoruso *et al*., 2010). PPARγ might, therefore, represent a novel target in various pathological conditions, especially chronic inflammatory diseases.

The aim of the second year of my PhD program was to evaluate the effects of monocyte-derived MPs on human monocytes and monocyte-derived macrophages (MDM) isolated from healthy donors, by evaluating the ability of MPs to trigger oxy-radical production, cytokine release (TNF-α and IL-6, as the most relevant pro-inflammatory cytokines) and NF-κB activation (that is, pivotal pro-inflammatory mechanisms) and to affect PPARγ protein expression. We also examined

the ability of PPARγ selective agonists (15d-PGJ and rosiglitazone, which exert important anti-inflammatory activities in human monocyte/macrophages; Amoruso *et al*., 2007, 2008, 2010) in modulating MPs-induced responses.

METHODS

Preparation of monocytes and monocyte-derived macrophages (MDM)

Human monocytes were isolated either from fresh buffy-coats, obtained by the local blood bank, or from heparinized venous blood (30-40 ml) of healthy non-smoker volunteers by standard techniques of dextran sedimentation, Hystopaque (density = 1.077 g cm^{-3}) gradient centrifugation (400 x *g*, 30 min, room temperature) and recovered by thin suction at the interface, as described (Amoruso *et al*., 2007, 2008, 2009a). Cells were washed twice in phosphate-buffered saline (PBS) and finally re-suspended in RPMI 1640 medium, supplemented with 5% heat-inactivated foetal bovine serum (FBS), 2 mM glutamine, 10 mM Hepes, 50 µg/ml streptomycin and 5 U/ml 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 phosphate-buffered saline (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 x 10^5 cells/well) were challenged with the compounds under study for different times (up to 24 h). Thereafter, the medium was replaced by the MTT solution (1mg/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, as described (Amoruso *et al*., 2010). A different number of cells were used according to the type of experiments (2 x 10^6 cells for Western blot experiments, 5 x 10^6 cells for EMSA assays, 0.5 – 1 x 10^6 cells for superoxide anion production and 1 x 10^6 cells for cytokine release).

Generation and characterization of monocyte-derived microparticles (MPs)

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

Supernatants from healthy donors were used in the experiments here reported and evaluated for MPs characterization, by assessing phosphatidylserine (PS) expression, Tissue Factor (TF) activity and $CD14^+$ elements.

PS positive MPs in each sample were detected using the Zymuphen MP-activity kit (Hyphen BioMed, Neuville-sur-Oise, France), according to manufacturer's instructions and expressed as PS equivalents. TF activity was measured in MPs generated in vitro from human monocytes by a one-stage clotting time as described (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 sec (Celi *et al*., 1994).

To characterize the MPs generated from monocytes, samples were analyzed by flow cytometry using a FACS Calibur (Becton Dickinson, Oxford, U.K.). 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 re-suspended human monocytes and 100 µl of MPs were added for 30 min in the dark on ice to the fluorescent dye-labelled antibody against surface marker $CD14^+$ PE (Phycoerythrin)-conjugated (Dako Cytomation, Denmark). CD14 expression positivity was defined on FL2 (PE fluorescence) *vs* FSC dot plot representation. Regions (R1) corresponding to monocytes and MPs were defined on forward *vs* 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.

In keeping with Scanu *et al*. (2008) and Carpintero *et al*. (2010), we also measured total proteins content, with a BCA assay, in the supernatant samples; this allows a quantitative measure, as well as a better evaluation of possible concentration-dependent effects of MPs. In order to ensure that the effects observed with our MPs preparations were not attributable to soluble mediators that may be present within supernatants, supernatants were ultra-centrifuged at 100,000 x *g* for 2 h (Neri *et al*., 2011). 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.

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

Adherent cells (0.5 – 1 x 10^6 cells/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 content, 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 maximal effective concentrations of the protein kinase C activator, phorbol 12-myristate 13-acetate (PMA; 10^{-6} M). In some experiments, cells were pre-treated with 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ; 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/10^6cells/30 min, using an extinction coefficient of 21.1 mM (Bardelli *et al*., 2005). Control values (e.g., basal production in the absence of stimuli) were subtracted from all determinations.

Cytokine release in monocytes and MDM

Human monocytes and MDM challenged with increasing concentrations of MPs or PMA 10^{-7} M for 24 h; this time was previously shown to ensure maximal cytokine release (Bardelli *et al*., 2005; Amoruso *et al*., 2010). Supernatants were collected and stored at - 80°C. TNF-α and IL-6 were measured by ELISA kits (R&D Systems, Minneapolis, USA) and the amount of each cytokine was expressed in pg/ml, as indicated by the manufacturer's instructions. The minimum detectable concentrations of human TNF-α and IL-6 were 1.4 pg/ml and 0.5 pg/ml, respectively. Control values (e.g., cytokine release from untreated, un-stimulated cells) were subtracted from all determinations. In some experiments, cells were pre-treated for 1 h with 15d-PGJ 10 μM or rosiglitazone 1 μM, in the absence or presence of the PPARγ antagonist 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; not shown).

Evaluation of NF-κB activation

The activation of NF-κB induced by MPs or PMA was evaluated by measuring its nuclear translocation (by electrophoretic mobility shift assay, 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. Densitometric analysis of NF-κB nuclear translocation was also performed, un-stimulated cells being = 1. In some experiments, cells were pre-treated with 15d-PGJ 10 μM or rosiglitazone 5 μM for 1 h and then challenged with MPs or PMA 10^{-6} M. At the concentrations used, neither 15d-PGJ 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 x 10^6 cells/well) were washed twice with ice-cold PBS, scraped off in RIPA buffer; when necessary, cell lysates were stored at -80°C. The determination of protein concentration was done with a 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 (that is, 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). Data are expressed as mean ± SEM of "*n*" independent experiments on cells isolated from different healthy donors. 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 in percentage, as in the case of cytokine and O_2^- inhibition, and EMSA quantification. Differences were considered statistically significant when $p < 0.05$.

RESULTS

Characterization of monocyte-derived MPs

MPs used in this study are generated from A23187-challenged human monocytes. To characterize these MPs, supernatants (100 µl) from un-stimulated or A23187-challenged monocytes have been evaluated for CD14 expression, Tissue Factor (TF) activity and phosphatidylserine (PS) equivalents concentration (Figures 1 and 2). **Figure 1** shows forward *vs* side angle light scatter dot plot and the CD14 positivity expression representation of un-stimulated monocytes re-suspended in 100µl of PBS (A) and 100 µl of 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.

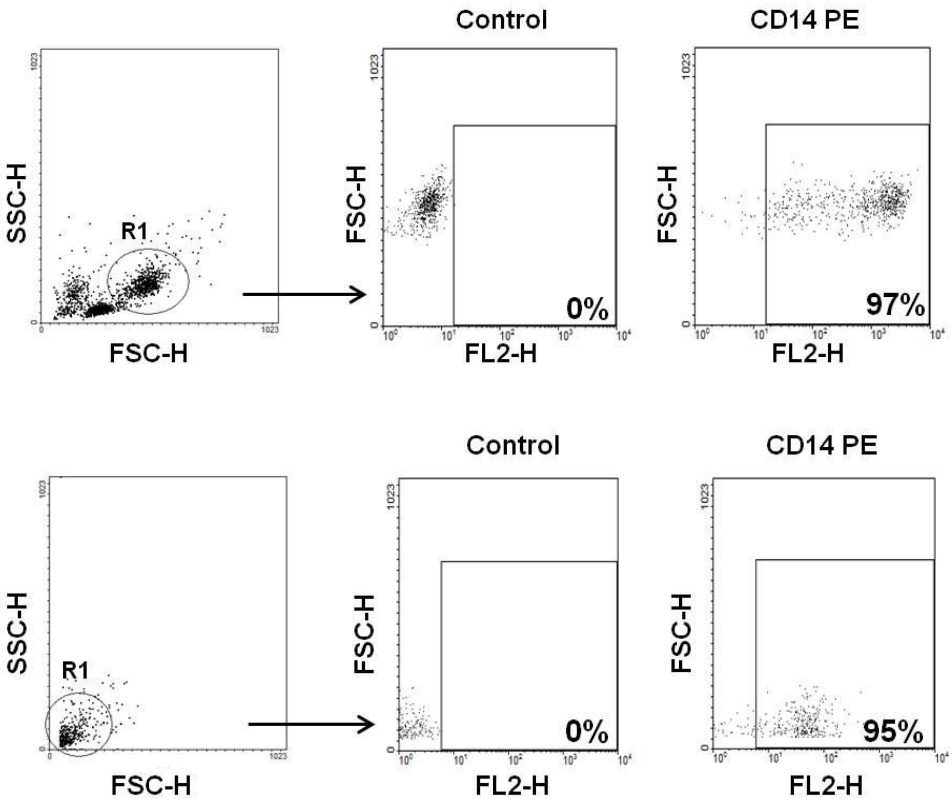

Fig.1 Flow cytometric analysis of monocytes-derived microparticles (**MPs**). Regions (R1) corresponding to monocytes (A) and MPs (B) were defined on forward (FSC) versus side angle light scatter (SSC) intensity dot plot representation. CD14 expression positivity was defined on FL2 (PE fluorescence) versus FSC dot plot representation.

Moreover, as reported in **Figure 2**, only supernatants from A23187-stimulated monocytes present an important TF activity (9824 + 1700 A.U.; *n* = 6) and PS concentration (2.04 + 0.4 nM; *n* = 6), thus confirming MPs formation. As an attempt to quantify MPs and in view of their possible concentration-dependent effects, we have evaluated the content of total proteins in 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 experimental assays performed, MPs were used in the range 0.1 - 30 µg/ml.

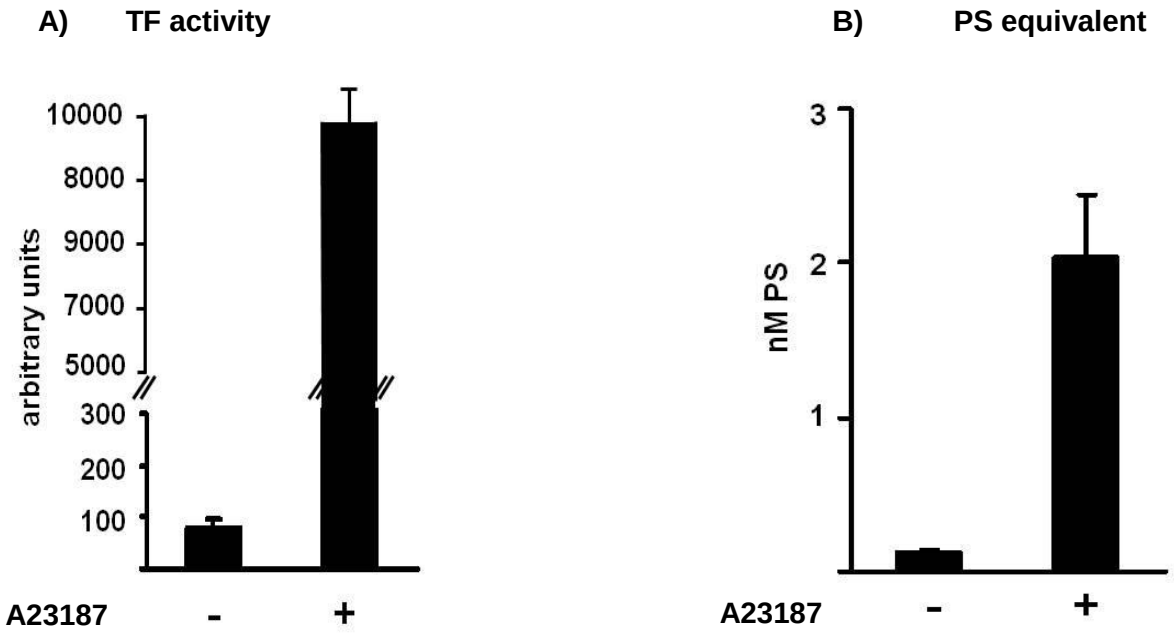

Fig.2 Characterization of monocyte-derived microparticles (MPs). A) **TF activity** in 100 µl of supernatant from un-stimulated monocytes and stimulate monocytes (with A23187, 12 µM). B) Corresponding concentration of **PS equivalents**. Data are mean ± SEM of 6 different MPs preparations.

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

To evaluated O_2^- production, monocytes and MDM were challenged with different stimuli for 30 min. Monocyte-derived MPs induce, in a concentration-dependent manner (0.1 – 30 µg protein content), O_2^- production in human monocytes and MDM (**Figure 3A**), with maximal effect at 10 - 30 µg. The EC_{50} values are 1.55 µg/ml of supernatant (containing MPs) in monocytes and 2 µg/ml in MDM.

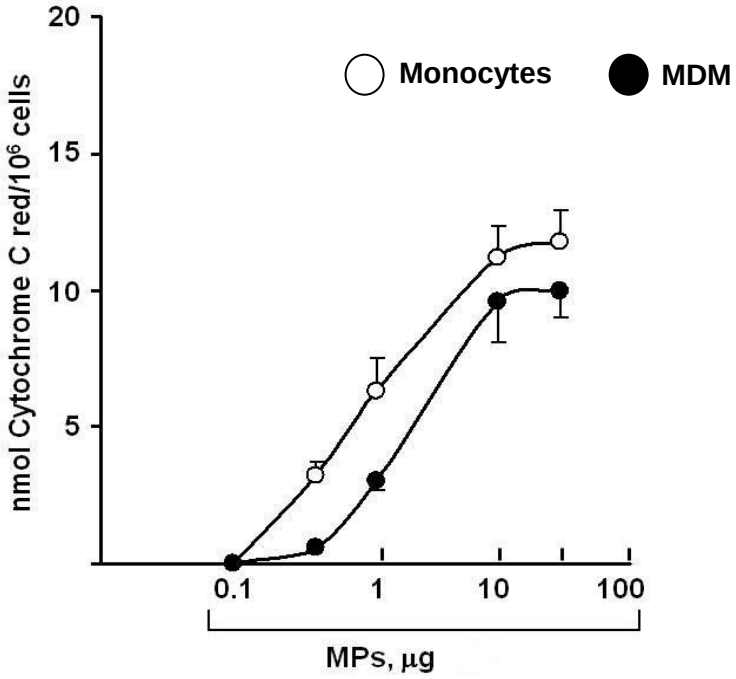

Fig.3A Monocyte-derived MPs induce superoxide anion (O_2^-) production in a concentration-dependent manner (0.1- 30 µg protein content), in **monocytes** and **MDM**. Data are mean ± SEM; *n* = 14 (monocytes), 8 (MDM).

As depicted in **Figure 3B,** MPs-induced oxy-radical production is significantly lower than that evoked by PMA 10^{-6} M (23.7 ± 4 nmol cytochrome C reduced/10^6 cells in monocytes and 43 ± 8 in MDM). Moreover, spontaneous O_2^- production (control) is minimal in both monocytes and MDM and ultra-centrifuged supernatants (from which MPs have been removed) or supernatants from un-stimulated monocytes do not affect respiratory burst. Conversely, the pellet from ultra-centrifuged samples induced O_2^- production, although less effectively that the original MPs. In order to demonstrate that there was no direct influence of the calcium ionophore in the oxy-radical production, we stimulated the cells with A23187 at 4 µM (that is the concentration resulting in the 300 µl of supernatant) and verified that, in this case, the oxy-radical production was minimal.

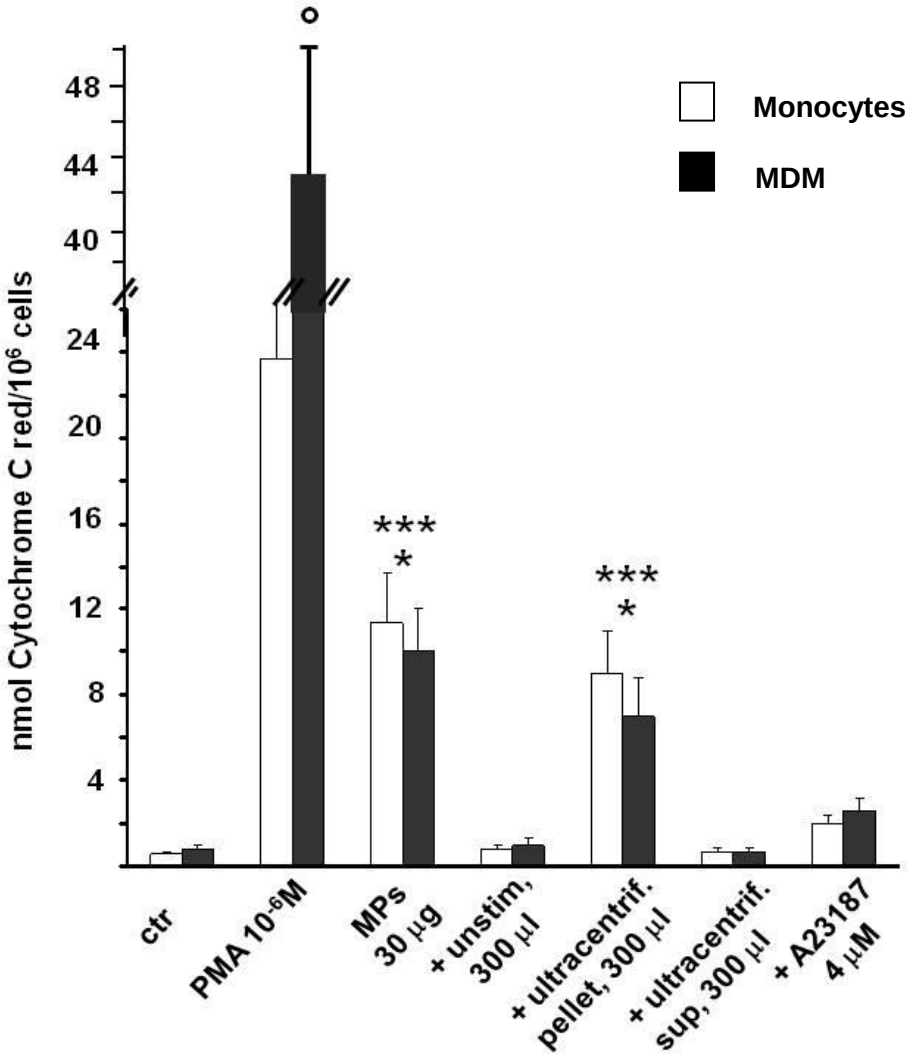

Fig.3B Superoxide anion (O_2^-) production induced by PMA (1 µM), MPs (30 µg protein content), supernatant from un-stimulated cells (300 µl), ultra-centrifuged pellets (300 µl) and supernatants (300 µl), and by A23187 (4 µM), in **monocytes** and **MDM**. Data are mean ± SEM; *n* = 14 for control, PMA and MPs; *n* = 3 for the other groups. °*p* < 0.05 *vs* monocytes; **p* < 0.05 *vs* PMA; ***p* < 0.05 *vs* control.

As expected, the endogenous PPARγ agonist, 15d-PGJ, 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 (76 ± 4 %; *n* = 5) than PMA-evoked one (95 ± 2 %; *n* = 5) (**Figure 3C**).

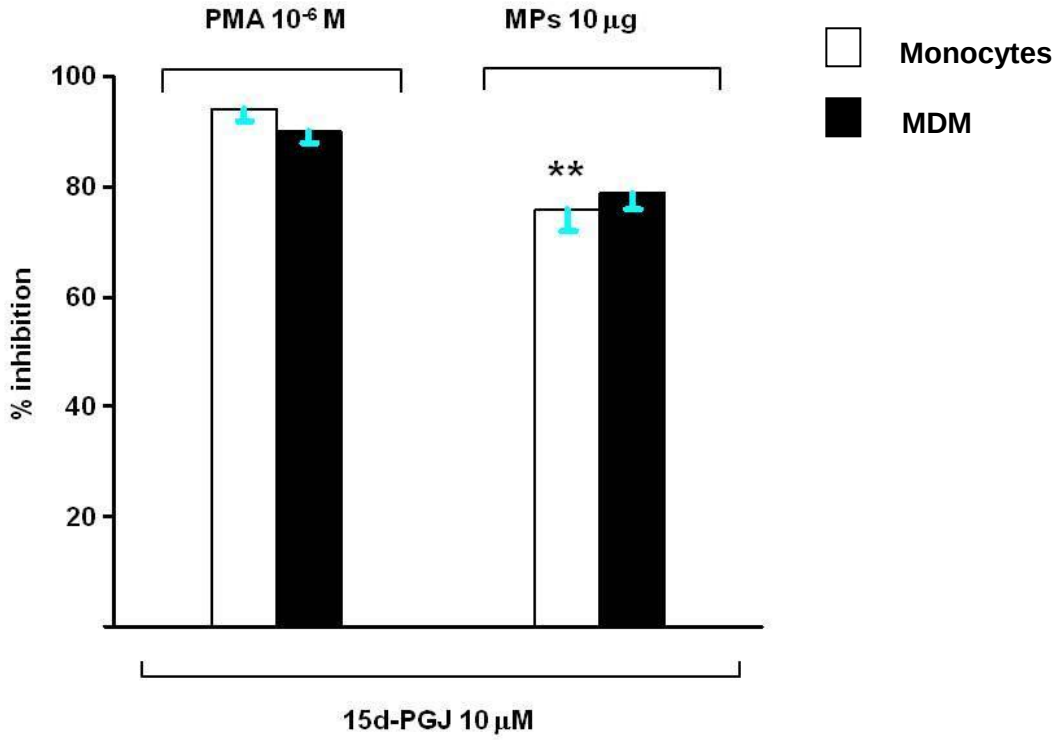

Fig.3C % inhibition of PMA- and MP- evoked O_2^- production by 15d-PGJ used at 10 µM, in **monocytes** and **MDM**. Data are mean ± SEM; *n* = 5; ***p* < 0.05 *vs* PMA.

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

As shown in **Figure 4**, PMA and MPs induce 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. MPs, evaluated in the range 1 - 30 µg protein content, exert concentration-dependent effects with maximal effects at 10 µg.

In human monocytes (**Figure 4A**), MPs release higher amounts of IL-6 as compared to TNF-α, whereas, for PMA, cytokine release were not significantly different. Basal cytokine release from un-stimulated human monocytes (TNF-α = 10 ± 4 pg/ml and IL-6 = 38 ± 14 pg/ml; *n* = 5) is subtracted from all determinations. Maximal release of TNF-α or IL-6 by MPs was observed at 10 µg protein content (91 ± 15 pg/ml for TNF-α and 348 ± 37 pg/ml for IL-6) and is enhanced by the PPARγ antagonist, GW 9662, used at 1 µM. Rosiglitazone (1 µM) and 15d-PGJ (10 µM), previously shown to exert maximal effects with no evident sign of cytotoxicity (Amoruso *et al*., 2008, 2009b, 2010), inhibit MPs-induced cytokine release (see also Table 1); this inhibition is completely reversed by GW 9662 (**Figure 4A**).

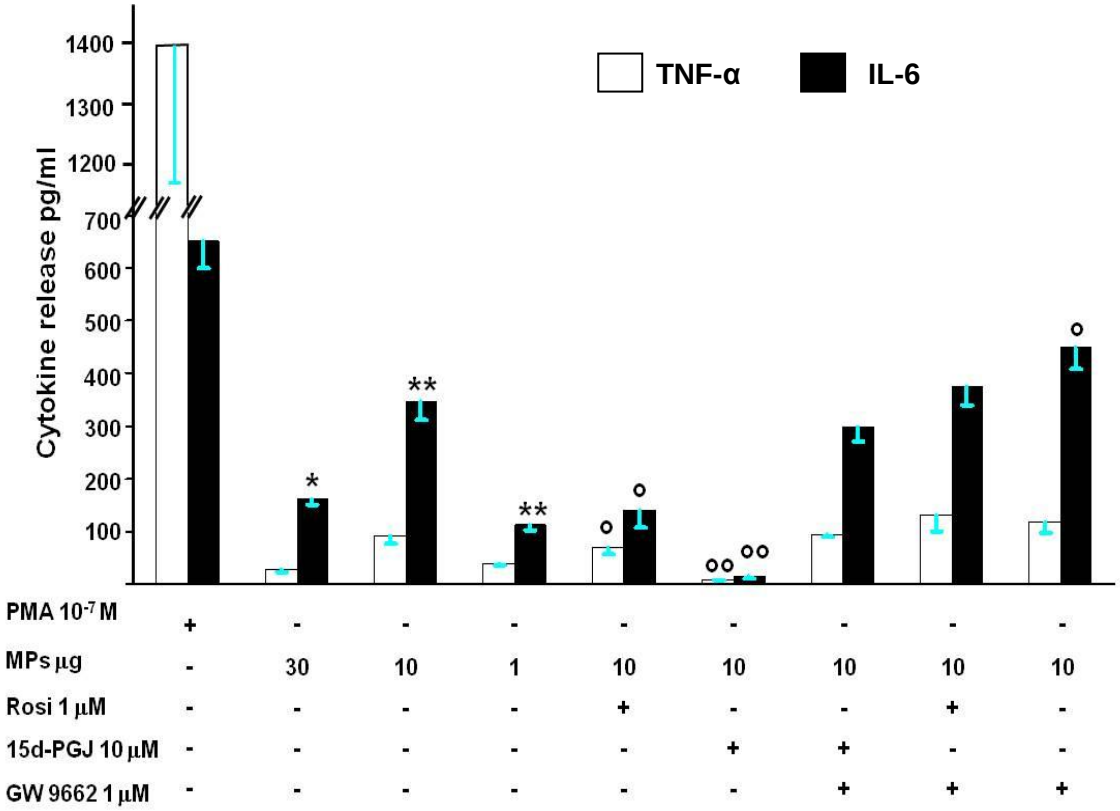

Fig.4A Monocyte-derived MPs induce cytokine production in **monocytes**. Concentration-dependent (1 - 30 µg) effects of MPs and modulation by PPARγ agonists (15d-PGJ 10 µM, rosiglitazone: Rosi, 1 µM) and antagonist (GW 9662, 1 µM). TNF-α and IL-6 release produced by PMA 10^{-7} M are shown for comparison. Data are means ± SEM; $n = 5$. *$p < 0.05$ *vs* TNF-α; **$p < 0.01$ *vs* TNF-α; °$p < 0.05$ *vs* MPs 10 µg; °°$p < 0.01$ *vs* MPs 10 µg.

In human MDM (**Figure 4B**), PMA and MPs induce TNF-α and IL-6 release (at lower amounts as compared to human monocytes) that is potently inhibited by the two PPARγ ligands (15d-PGJ exerting higher effects than rosiglitazone). This inhibition was reversed by GW9662. In this case too, basal cytokine release (TNF-α = 20 $\pm$ 5 pg/ml, IL-6 = 25 $\pm$ 10 pg/ml; $n = 5$) is subtracted from all determinations.

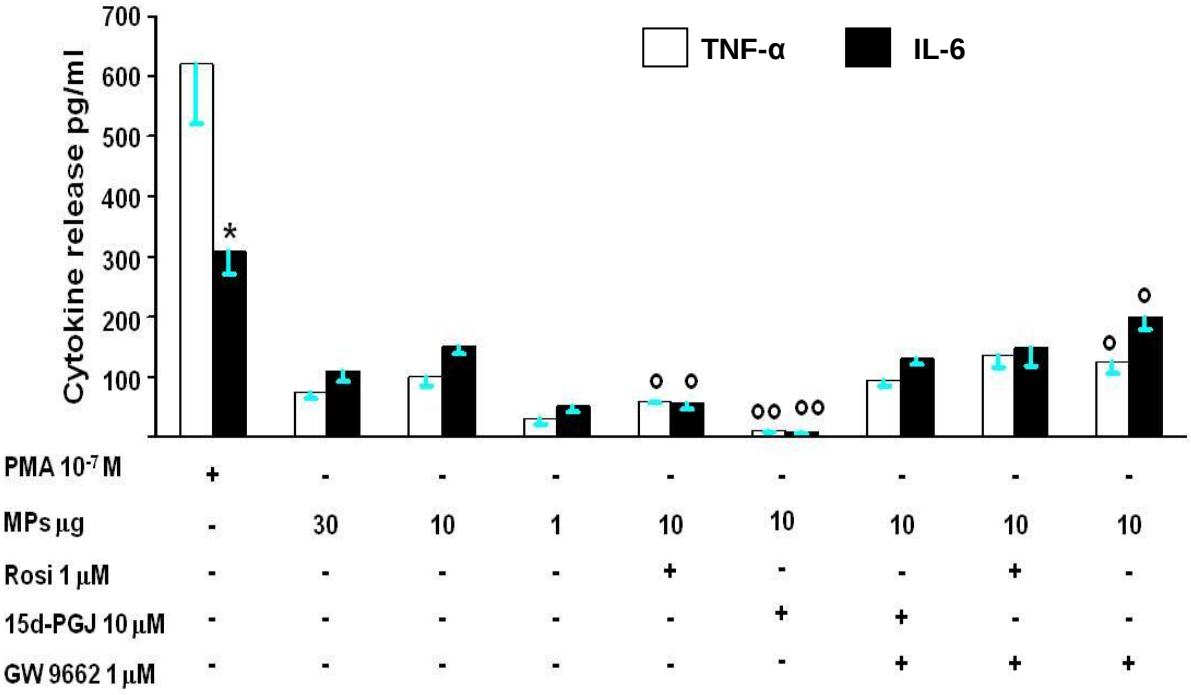

Fig.4B Monocyte-derived MPs induce cytokine production in **MDM**. Concentration-dependent (1-30 µg) effects of MPs and modulation by PPARγ agonists (15d-PGJ 10 µM, rosiglitazone: Rosi, 1 µM) and antagonist (GW 9662, 1 µM). TNF-α and IL-6 release produced by PMA 10^{-7} M are shown for comparison. Data are means ± SEM; $n = 5$. *$p < 0.05$ *vs* TNF-α; **$p < 0.01$ *vs* TNF-α; °$p < 0.05$ *vs* MPs 10 µg; °°$p < 0.01$ *vs* MPs 10 µg.

As reported in **Table 1**, some differences are observed concerning the inhibitory potential 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. In keeping with previous observations (Amoruso *et al*., 2008, 2009b, 2010), at maximally effective concentrations, 15d-PGJ often exerts a significant higher inhibition than rosiglitazone, with no major differences (except in the case of TNF-α release in human monocytes) related to the stimuli (PMA or MPs). In these experiments, the inhibition by rosiglitazone on MPs-induced cytokine release was usually lower (although not always reaching statistical significance) than the PMA-induced cytokine release. This is particularly evident in human monocytes, in which rosiglitazone inhibits by more than 60% PMA-induced TNF-α release, while it reduces MPs-evoked release by only 27% (**Table1**).

	% inhibition on MPs 10 µg		% inhibition on 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	92 ± 1°°	96 ± 1°°	95 ± 2°°	82 ± 4°°
MDM (*n*=5)				
Rosi	28 ± 5	30 ± 3	40 ± 8	42 ± 7
15d-PGJ	40 ± 6	54 ± 5°°	52 ± 3	58 ± 5

Table 1. Rosiglitazone (Rosi) 1 µM and 15d-PGJ 10 µM inhibit PMA- and MPs-evoked cytokine release in **monocytes** and **MDM**. Cells were pre-treated 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. Data are means ± SEM; *n* = 5; ***p* < 0.01 *vs* TNF-α; °°*p* < 0.01 *vs* rosiglitazone; $^{§§}p$< 0.01 *vs* MPs.

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

Figure 5 shows NF-κB activation in monocytes and MDM, challenged with PMA and MPs. In un-stimulated monocytes, DNA binding of NF-κB is minimal, although detectable, whereas it is considerably increased following challenge with PMA or MPs (**Figure 5A**). Nuclear translocation of the transcription factor is maximal when monocytes are stimulated by PMA 10^{-6} M for 1 h (> 3-fold increase *vs* control). MPs, evaluated at different concentrations (1, 5, 10 and 30 µg protein content), also enhance NF-κB nuclear migration, with maximal effect at 10 µg (2.4 ± 0.3 *vs* control). Previous reports (Amoruso *et al*., 2009b, 2010; Neri *et al*., 2011; Tesse *et al*., 2008) documented the ability of PPARγ agonists to inhibit NF-κB activation. When used at the near maximal concentration (5 µM), rosiglitazone potently inhibits MPs-triggered NF-κB translocation, with values similar to un-stimulated monocytes in densitometric evaluations, while it is modestly effective in reducing PMA-induced translocation (**Figure 5A**).

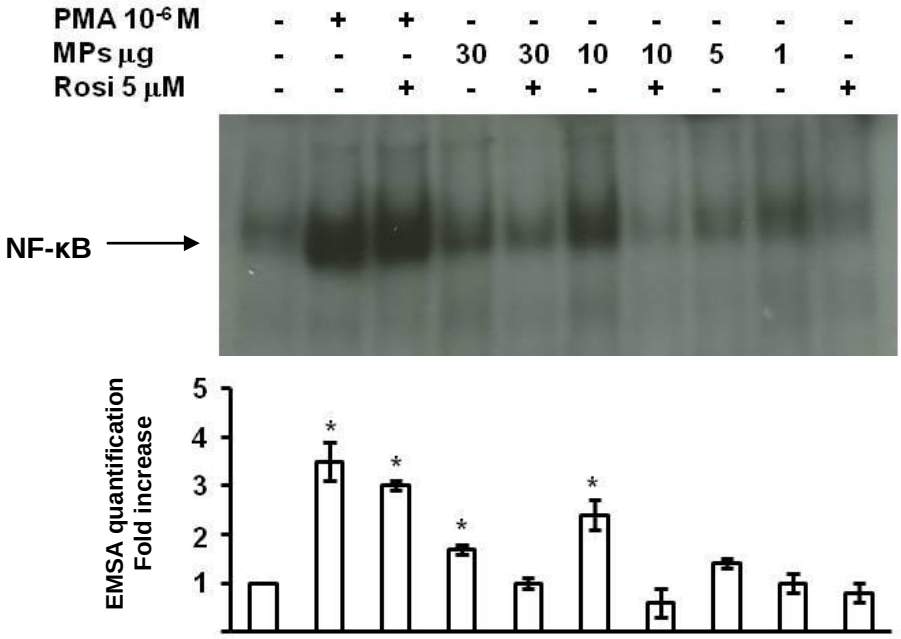

Fig.5A Monocyte-derived MPs induce NF-κB activation in **monocytes**. Concentration-dependent (1 - 30 µg) effects of MPs and modulation by rosiglitazone (Rosi, 5 µM). The effects produced by PMA 10^{-6} M are shown for comparison. Histogram below the blot is densitometric analysis for EMSA quantification (control, that is un-stimulated cells = 1). Data are means ± SEM; $n = 4$.*$p < 0.05$ *vs* control.

In human MDM, MPs exert a concentration (1 - 30 µg)-dependent effect and, at the highest 10 and 30 µg protein content, were even more potent than PMA in evoking NF-κB nuclear migration (**Figure 5B**). EMSA quantification (setting un-stimulated MDM = 1) revealed that NF-κB translocation evoked by MPs 10 µg was almost double of that one of PMA (fold increase: PMA = 3.1 ± 0.4, MPs = 5.96 ± 0.6) (**Figure 5B**). In MDM, rosiglitazone potently reduces NF-κB activation evoked by MPs, while it is less effective against PMA-induced activation (**Figure 5B**).

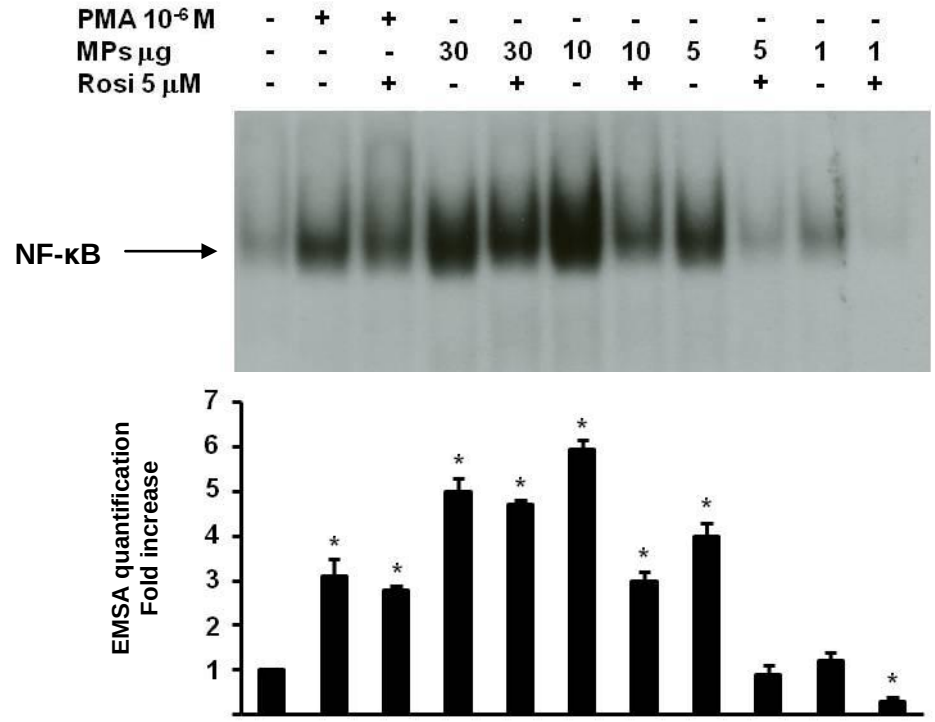

Fig.5B Monocyte-derived MPs induce NF-κB activation in **MDM**. Concentration-dependent (1 - 30 µg) effects of MPs and modulation by rosiglitazone (Rosi, 5 µM). The effects produced by PMA 10^{-6} M are shown for comparison. Histogram below the blot is densitometric analysis for EMSA quantification (control, that is un-stimulated cells = 1). Data are means ± SEM; $n = 4$. *$p < 0.05$ *vs* control.

As depicted in **Figure 6**, in human monocytes, NF-κB translocation induced by MPs (10 or 30 µg protein content) is significantly higher than that evoked directly by stimulation with the calcium ionophore A23187 (used at 4 µM, corresponding to 300 µl aliquot of supernatant) and, as expected, supernatant from un-stimulated monocytes is devoid of activity. In order to confirm that MPs, rather than soluble molecules present within the supernatant, were responsible for NF-κB nuclear translocation, we performed some experiments with ultra-centrifuged materials. As expected, supernatant from ultra-centrifuged materials was devoid of activity. The pellet from ultra-centrifuged samples still induced some NF-κB translocation, but it was less effective, as already observed for superoxide anion production (**Figure 6**). 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 concentration displaying a lesser effect than the 10 µg/ml

concentration, it is conceivable that a more elevated concentration (as in the case of ultra-centrifuged pellet) could exert a lower activity.

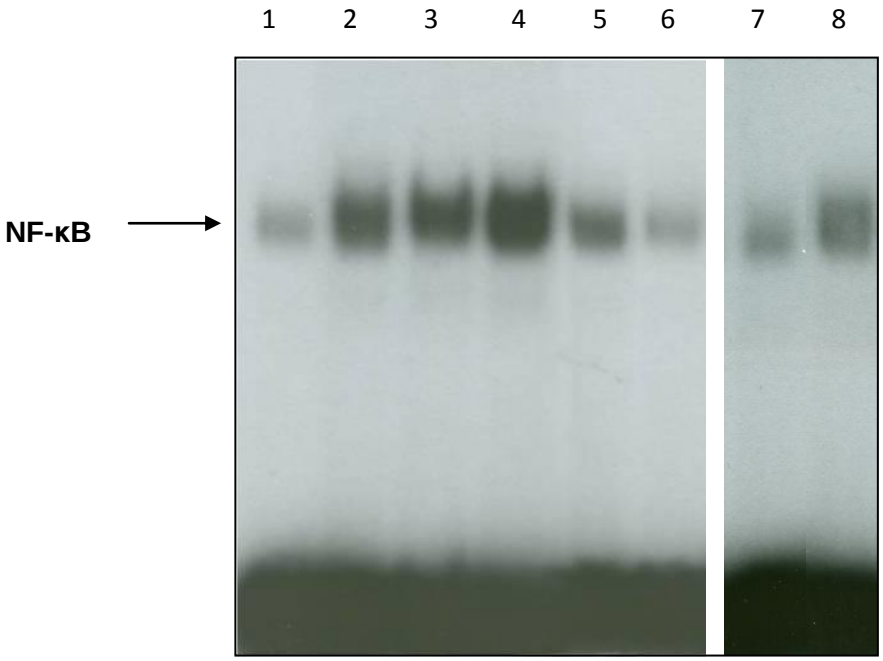

Fig.6 In **monocytes**, monocyte-derived MPs, but not ultra-centrifuged supernatants, induce NF-κB activation. Lane 1: un-stimulated monocytes; lane 2: +PMA 1 μM; lane 3: + MPs 30 μg; lane 4: + MPs 10 μg; lane 5: + ultra-centrifuged pellet (300 μl) from A23187-stimulated monocytes; lane 6: + ultra-centrifuged supernatant (300 μl) from A23187-stimulated monocytes; lane 7: + supernatant (300 μl) from un-stimulated monocytes; lane 8: + A23187 4 μM.

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 is up-regulated during differentiation to MDM, as previously reported (Amoruso *et al*., 2007, 2009a). Two PPARγ agonists (rosiglitazone and 15d-PGJ) enhance PPARγ protein expression about two-fold in both cell types, in good accordance to previous reports (Amoruso *et al*., 2007, 2009a). Interestingly, when evaluated at 10 μg protein content, MPs induce PPARγ protein expression in both monocytes and MDM, with minor efficacy compared to the effects exerted by selective ligands (**Table 2**). A representative Western blot is provided in **Figure 7**.

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

Table 2 PPARγ protein expression in **monocytes** and **MDM**. PPARγ protein expression was semi-quantified by measuring the ratio between PPARγ expression and β-actin expression. Data are means + SEM; *n* = 5; **p* < 0.05 *vs* un-stimulated cells of the same type; °°*p* < 0.01 *vs* control monocytes.

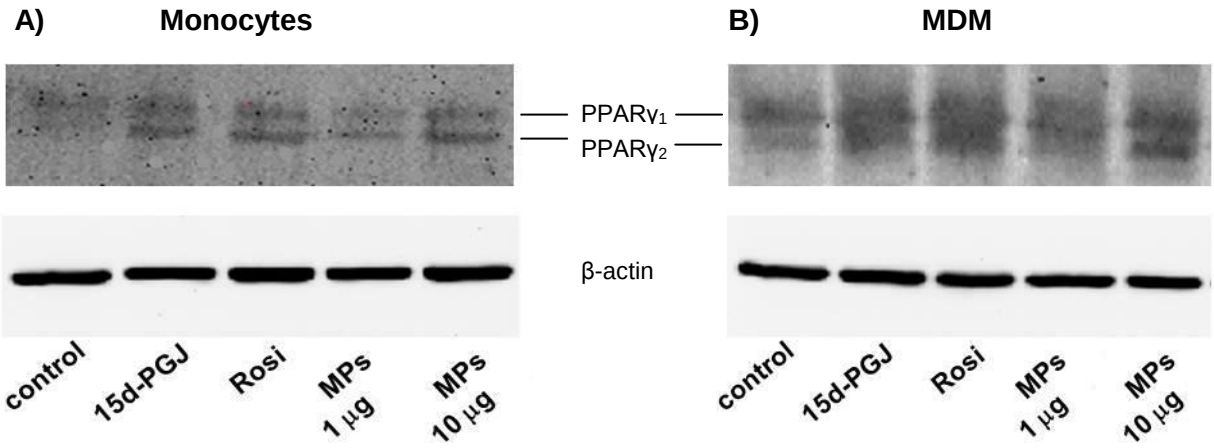

Fig.7 Monocyte-derived MPs induce PPARγ protein expression in **monocytes** (A) and **MDM** (B). Cells were challenged with 15d-PGJ 10 µM, rosiglitazone (Rosi, 1 µM) or MPs (1 or 10 µg protein content) for 6 h. This representative blot shows PPARγ (both 1 and 2 isoforms) and β-actin expression.

DISCUSSION

The results obtained during the second year of my PhD program demonstrated, for the first time, that microparticles (MPs) generated from A23187-stimulated human monocytes deeply affect human cells of the same lineage, demonstrating therefore an autocrine action. In fact, we show that monocyte-derived MPs induce oxy-radical production, cytokine release, NF-κB activation and PPARγ protein expression in both human monocytes and macrophages (MDM).

MPs can be generated from several cell types (e.g. platelets, endothelial cells, monocytes, macrophages, B- and T- cells) and present cell surface markers and cytoplasmatic components of their precursor cells (Distler *et al*., 2005a; Dalli *et al*., 2008). Therefore, depending on their origin, MPs differ in size (diameter 0.1-1 μm), protein and lipid composition, and effects. There are two well-known cellular processes that can lead to the formation of MPs: cell activation and apoptosis (VanWijk *et al*., 2003). For instance, MPs release can be induced by collagen, calcium ionophore A23187, LPS, cytokines (TNF-α or IL-1) or chemical compounds, such as phorbol myristate acetate (PMA) and ionomycin (Ardoin *et al*., 2007). We prepared MPs as previously described (Neri *et al*., 2011), by treating monocytes with A23187 12 μM, that induces a rapid and complete MPs release, a method largely used (Satta *et al*., 1994). Indeed, 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 (Cerri *et al*., 2006).

The identification of MPs is, generally, based on the size and the surface markers expression, and flow cytometry is the most commonly method used to analyze the features of MPs. However, their characterization is not a standardized procedure: we have addressed this item by evaluating percentage of $CD14^+$ elements, TF activity, PS equivalents' concentration, as well as protein concentration in the samples. In our MPs preparations, 100 μl of supernatants from A23187-challenged monocytes correspond to 10 μg of protein content (that, in our experiments, usually ensures maximal effects) and 2 nM equivalents of PS, in good agreement with other reports (Carpintero *et al*., 2010; Scanu *et al*., 2008; Tesse *et al*., 2008). In fact, Scanu *et al*. (2008) and Carpintero *et al*. (2010) report maximal effects with 30 and 6 μg protein content for T cell-derived MPs, respectively, while Tesse *et al*. (2008), in experiments on vascular reactivity, use 30 nM PS equivalents of T cell-derived MPs.

Several studies have demonstrated that MPs originated even from different cell types exert pro-inflammatory activities in monocyte/macrophages (Barry *et al*., 1998; Carpintero *et al*., 2010; Distler *et al*., 2005b; Scanu *et al*., 2008). Our novel finding is that these effects are reproduced by monocyte-derived MPs too. These results open a new scenario suggesting that MPs not only

behave as relevant mediators in cell-to-cell communication or signalling over long distances, but also represent a key component for autocrine stimulation.

An interesting finding of our study is that monocyte-derived MPs exert concentration-dependent effects. 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 substantially agree and further improve 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, which peaks at 10 - 30 μg protein content.

Our results also provide evidence that monocyte-derived MPs induce, in a concentration-dependent manner, TNF-α and IL-6 release from human monocyte/macrophages, and also demonstrate that rosiglitazone and 15d-PGJ reduce these effects. These data are supported by other previous works (Mesri and Altieri, 1999; Tesse *et al*., 2008; Neri *et al*., 2011). In fact, Tesse *et al*. (2008) previously documented that rosiglitazone, used at 5 μM, prevents the increase of IL-6, IL-1β and IL-8 mRNA expression induced by T-cell derived MPs in human endothelial cells. Moreover, in human lung epithelial cells, Neri *et al*. (2011) demonstrated that monocyte-derived MPs increased IL-8 and MCP-1 synthesis, which was inhibited by rosiglitazone and 15d-PGJ, likely *via* interaction with PPARγ. Since these molecules have the potential to exert biological effects independently 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 reverts the effects of the two PPARγ agonists, we confirm the direct involvement of this receptor in the MPs effects on cytokine release.

Activation of PPARγ by selective agonists (e.g. 15d-PGJ and rosiglitazone) induce anti-inflammatory effects, at least in part through suppression of the NF-κB pathway, resulting in down-regulation of some inflammatory molecules, such as TNF-α, IL-6 and IL-1β (Amoruso *et al*., 2010). Indeed, PPARγ can physically interact with p65 subunit of NF-κB, so preventing its nuclear translocation (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 induce NF-κB nuclear translocation, which is significantly reduced by PPARγ agonists, in good agreement with previous observations (Neri *et al*., 2011; Tesse *et al*., 2008). Moreover, MPs-induced NF-κB nuclear migration is similar to that evoked by PMA and significantly higher than that induced by the direct A23187 stimulation.

Furthermore, monocyte-derived MPs stimulate (about twofold) PPARγ protein expression in human monocytes and MDM, with maximal effects lower than those induced by the selective ligands rosiglitazone and 15d-PGJ. Therefore, these results further extend previous data

describing anti-inflammatory properties for MPs (Dalli *et al*., 2008; Köppler *et al*., 2006; Gasser and Schifferli, 2004). As an example, the transfer of tumour cells-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 (Köppler *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. As known, PPARγ is expressed in human monocytes, and its amount increases in human macrophages (Amoruso *et al*., 2007, 2008).

In conclusion, the results obtained in this second year of my PhD program, originally demonstrate that monocyte-derived MPs have relevant pro-inflammatory effects, that are reduced by PPARγ agonists, in human monocytes and MDM. This data suggests that monocyte-derived MPs are relevant to autocrine stimulation in human cells. Moreover, our results indicate a novel activity, which is enhancement of PPARγ protein expression, for monocyte-derived MPs, supporting a possible anti-inflammatory loop.

MPs represent a disseminated storage pool of bioactive effectors and they differ qualitatively and quantitatively, depending on parent cell types and on cellular process triggering their release. Therefore, MPs' properties need to be carefully evaluated, according to their nature and peculiar recipient cells, given the possibility of MPs dual effects.

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ABSTRACTS

- Anti-inflammatory Effects of the CINOD NCX 429 in Human Monocytes and Macrophages. **D Federici Canova**, A Amoruso, LG Fresu, J Padron, M Bolla, S Brunelleschi.
  35th National Congress of "Italian Society of Pharmacology- SIF" - **Bologna** (September 14-17 2011).

- A possible involvement of Monocyte-derived Microparticles in alveolar macrophages inflammatory response.
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  35th National Congress of "Italian Society of Pharmacology- SIF" - **Bologna** (September 14-17 2011).

CONGRESS PARTECIPATIONS

- Regional Congress of "Italian Society for the study of Atherosclerosis - SISA" (Section "Lombardia") - **Milan** (April, 15 - 16, 2011).
- 35th National Congress of "Italian Society of Pharmacology- SIF" - **Bologna** (September 14 - 17, 2011).

SEMINARS (Dept. Medical Science 2010-2011)

- *Le cellule NK: dalla biologia alla terapia di leucemie ad alto rischio* Prof. L. Moretta (November, 29th 2010)
- *Targeting Lactate- Fueled respiration in cancer: a new therapeutic opportunity?* Prof. P. Sonveaux (December, 1st, 2010)
- *Bone and DDS: development of bioactive artificial bone with drug delivery ability* Dr. M. Otsuka (January, 27th, 2011)
- *DNA-complex releasing system by apatitic cement for gene therapy* Dr. T. Ito (January, 28th, 2011)
- *Ferritins: ancient proteins with novel unexpected features* Dr S. Levi (April, 12th, 2011)
- *Metabolic syndrome: the gastroenterologist point of view* Dr E. Bugianesi (April, 13th, 2011)
- *Nanotecnologie applicate alla medicina: le nuove frontiere della nano medicina* Prof. G. Peluso (April, 14th,2011)
- *Reverse vaccination in autoimmune diseases?* Prof. G. Filaci (May, 3rd, 2011)
- *Innate Immunity and the pathogenicity of inhaled microbial particles.* Prof. H. Wolff (May, 9th, 2011)
- *Farmacologia dell'aterosclerosi* Prof. A. Corsini (May, 13th, 2011)
- *Mechanisms and Models of TDP-43 Proteinopathies* Prof. L. Petruccelli (May, 19th, 2011)
- *Iron Management in the Hepcidin Era* Prof. SR. Ellis (May, 25th, 2011)
- *Molecular pathogenesis and biomarkers of Parkinson's disease* Prof. M. Fasano (June, 17th, 2011)
- *Importance of pathobiology in rheumatoid arthritis pathogenesis, disease evolution and response to treatment (The PEAC Project)* Prof. C. Pitzalis (June, 24th, 2011)
- *Management of high risk chronic lymphocytic leukemia* Prof. T. Zenz (June, 30th, 2011)
- *Hypoxia, angiogenesis and liver fibrogenesis* Prof. M. Parola (July, 1st, 2011)

OTHER ACTIVITIES:

- Frontal lessons: “Degenerative diseases” (Prof. Albano, Prof. Corrado, Prof. Comi)
- COREP seminars
- Journal Club (Dept. Medical Sciences)