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## Characterization of new loops establishing between human Malignant Mesothelioma (MM) and monocytes/macrophages and evidences about their possible pathological implications

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To the beloved memory of Prof. P.M.

Baldini.

A mother, a friend and a guide.

We all miss you.

# Author's publications

**Izzi V.,** Chiurchiù V., Doldo E., Albonici L., Manzari V., Bei R. and Modesti A. "Interleukin-17 (il-17) is produced by malignant mesothelioma-conditioned immune cells and promotes tumor growth and invasiveness." *submitted*

**Izzi V.**, Chiurchiù V., D'Aquilio F., Palumbo C., Tresoldi I., Modesti A. and Baldini P.M. "Differential effects of malignant mesothelioma cells on THP-1 monocytes and macrophages." *International Journal of Oncology;* 2009; *34(2):543-50.*

**Izzi V.**, Chiurchiù V., D'Aquilio F., Martino A., Tresoldi I., Modesti A. and Baldini P.M. "Endomorphin-1 (EM-1) inhibits the activation and the development of a hyporesponsive-like phenotype in lipopolysaccharide (LPS)-stimulated THP-1 monocytes." *International Journal of Immunopathology and Pharmacology,* 2008*;* 21(4):833-843

Chiurchiù V., **Izzi V.**, D'Aquilio F., Carotenuto F., Di Nardo P., Baldini PM. "Brain Natriuretic Peptide (BNP) regulates the production of inflammatory mediators in human THP-1 macrophages." *Regulatory Peptides*, 2008; 148(1-3):26-32.

# INDEX

**Abstract**

Malignant mesothelioma (MM) is a tumor arising on body serousal membranes. It is regarded to as a highly fatal neoplasm, and more than a hundred clinical trials on the run and an expected 150-fold increase in peak incidence within the year 2050 should give an idea of the problem MM represents. Plus, although issues are still pending about the carcinogenic mechanisms leading to the development of such a neoplasm, evidences have been accumulated for its significant immunomodulatory activity.

Despite this fact, however, many molecular mechanisms are still lacking to explain the interactions between mesothelioma and cells belonging to the monocyte/macrophage lineage. These cells, in fact, are likely to play a crucial role in mesothelioma pathogenesis, and their accumulation in mesothelioma foci is very likely to represent a negative prognostic factor.

Basing on these considerations, the aim of my research was to focus on the crosstalk between immune cells and malignant mesothelioma in order to enlighten hierarchical relationships driving both monocyte/macrophage deregulation and the establishment of tumor-supporting activities in immune cells.

In this context, co-cultures and conditioned media have been used to allow interactions between malignant and immune cells, and data have been collected demonstrating different mechanisms determining tumor-induced physiopathological skewing of human monocytes and macrophages phenotypes and functions which clearly support active tumor growth and invasiveness.

# *Introduction and Aim*

## Overview of Malignant Mesothelioma

Malignant mesothelioma (MM) is a tumor deriving from mesothelial cells which form a single cell layer lining the pleural, peritoneal and pericardial spaces as a serousal membrane. MM is a highly fatal neoplasm which primary targets the pleural and peritoneal spaces and, less often, the pericardial spaces [1]. The gross appearance of MM features multiple well-defined nodules, with a generalized thickening of the serousal space and an invariable association with effusions of different degrees [2,3]. There are three major morphological presentations of MM, which are generally referred to as epithelioid, biphasic (or mixed) and sarcomatoid types [4]. Most MM are composed of round epithelial-like cells, with a general hystopathologic presentation in papillae and pseudoacini (sometimes presenting as plaques) [5]. To date, however, many different histological variants have been defined varying from the typical organization of epithelioid tissues [6]. Among these variants, spindle cell or sarcomatoid mesothelioma

(in which the cells are bundled in nodules) [7], desmoplastic mesothelioma (a subvariant form of sarcomatoid mesothelioma characterized by abundant fibrous tissue deposition) [8], lymphohistiocytoid mesothelioma (another form of sarcomatoid mesothelioma with unusual histiocyte-like morphology and abundant lymphocytes) [9], deciduoid mesothelioma (in which the cells present a ground glass cytoplasm resembling decidual cells) [10], squamous mesothelioma (also known as pleural squamous cell carcinoma) [11,12] and small cell variants [13] have been described so far. Suggested diagnosis of MM should be based on four different markers, chosen both as “positive” and “negative” ones, in order to exclude epithelial carcinomas of the organs enwrapped. At the moment, the best markers association is thought to include calretinin and cytokeratin 5/6 (or WT1) as positive markers together with CEA and MOC-31 (or B72.3) as negative ones [14,15]. Other markers such as N- and E-cadherin, CD445, mesothelin, HBME-1 and HMFG-2 [14,16,17] are of a limited diagnostic usefulness, and

recent findings about osteopontin, soluble mesothelin, and megakaryocyte potentiating factor (MPF) still need clinical evaluation [18]. Discrimination among different subtypes can be aided by additional cytological markers, such as vimentin, keratin and smooth muscle actin in sarcomatoid variants or neuron-specific enolase in small cell mesotheliomas [13,19-21]. Even though these markers greatly aided the diagnosis of MM, particularly in differentiating sarcomatoid MM from lung adenocarcinomas, a great difficulty is recognized still in discriminating MM from other hyperplasias, such as reactive mesothelial hyperplasia or mucoepidermoid carcinoma of the pleura [22-24]. Cytogenetic and molecular studies identified several characteristic alterations further aiding MM diagnosis. Although MM karyotypes are defined as complex, in fact, it has emerged that MM cells do not exceed five chromosomal abnormalities per cell, while adenocarcinomas typically have more than twenty events per cell. Moreover, chromosomal abnormalities are clearly non-random, deletional

hotspots being localized within the long arms of chromosome 22 and 6 and within the short arms of chromosomes 1, 3 and 9 [25]. Chromosomal deletions are likely to reflect the importance of gene loss in the pathogenesis of virtually all types of MM. Precise deletional targets have not been fully elucidated so far, major exceptions being CDKN2A and CDKN2B genes on 9p and NF2 on 22q [26]. The genomic loss or inactivation of CDKN2A and CDKN2B is a common mechanism in human cancers. These genes, in fact, encode proteins which block the cell cycle by inhibiting the CDK4 checkpoint kinase. As a consequence of CDKN2A/B loss, then, CDK4-cyclin D1 complexes are in the active conformation and phosphorylate their downstream effector retinoblastoma protein (Rb), inactivating it [27]. More interestingly, NF2 protein is inactivated in almost all mesotheliomas bearing chromosome 22 deletions, which are about 75% of all known mesotheliomas [28]. NF2 protein has been identified by the study of different neoplasias, such as neurofibromatosis type 2 and benign schwannomas

which are a typical feature of that syndrome. NF2 (currently termed Merlin) belongs to a family of moesin-ezrin-radixin-like protein which act as a membrane stabilizing protein, probably coupling membrane receptors with the cytoskeleton. Although its physiological role has not been clearly defined so far, evidences have been provided for its possible role in hyperplasias as an activator of Met and PDGF receptor kinases and an inhibitor of PI3 kinase [29].

Aetiology of malignant mesothelioma is undoubtly a major point of interest for such a neoplasia. MM were infrequently diagnosed before 1960, a time at which a dramatic MM increase was observed in asbestos miners [30]. Subsequent epidemiological studies confirmed the close relationship between MM and asbestos, this tumor common among diverse human groups being somehow exposed to asbestos, such as native Americans (exposed during the production of silver jewellery), carpenters, plumbers and electricians (exposed to asbestos insulation materials) and villagers of central Turkey

(exposed to inhalation of erionite, a structural mimic of asbestos) [1]. Since the latency period between asbestos exposure and MM presentation is typically longer than 30 years, a major health alarm is to be expected with MM because of the unregulated use of asbestos in many countries until 1970. This is confirmed by a registry of MM death in England, Scotland and Wales maintained since 1968: in these populations, deaths rose from 154 in late 60's to 1848 in early 2001 [31,32]. Asbestos belong to a family of magnesium and calcium silicates able to withstand extremely high temperatures. The thermoresistant properties of asbestos determined its modern widespread as insulation material for buildings and in heat-resistant materials in engine parts and brakes. Asbestos fibers are divided in two groups, depending on their conformation: wavy fibers are called serpentines, while straight fibers are called amphiboles and more often implicated in oncogenesis [33]. The oncogenic mechanism of asbestos fibers seem to be multifactorial, and to rely on both mesothelial and immune cells. As for

mesothelial cells, asbestos is thought to induce genotoxic damages, by breaking off chromosome pieces which are then separated by the mitotic spindle apparatus with a consequent random loss of genes. Obviously, when loss occurs within the above-described chromosomal regions, MM cells are likely to be generated [33]. Moreover, asbestos is also accounted for non-genotoxic carcinogenic damages, like the activation of the AP-1 – TNF-$\alpha$ – NF-kB autocrine axis leading to survival to genotoxic damages and helping aberrant cells to spread themselves along the tissue [34]. The contribute of immune cells to asbestos-induced MM generation is supposedly of an equal impact: the asbestos-induced insult to mesothelial integrity, in fact, is recognized by infiltrating leukocytes, which are mainly represented by activated macrophages. Under the pressure of mesothelium-derived inflammatory factors and the engulfment of asbestos fibers themselves, these cells are expected to release high amounts of classical inflammatory cytokines such as TNF-$\alpha$ and IL-1, setting a chronic inflammation on by

their inability to degrade the fibers. Unfortunately, these cytokines have a tumor-supporting role too, which also couple to the genotoxic damage which could affect mesothelial cells by the release of reactive oxygen intermediates by engulfed macrophages [34-36]. Unfortunately, modern regulations on asbestos usage in the world are not expected to eradicate MM, since only 10% of total neoplasms occur in asbestos-exposed subjects, clearly evidencing that other carcinogenic factors may be involved [37,38].

Among these carcinogenic or co-carcinogenic factors, a major controversy there is about the contribution of the oncogenic virus SV40. In 1994, it was demonstrated that Syrian hamsters developed mesotheliomas when intrapleurally injected with wild-type SV40, and it was also demonstrated that SV40-like sequences could be indentified by PCR in primary human MM [39]. However, different authors reported that the amplification of SV40-like sequences could depend on the specific primers used for PCR, together with a weak and non-nuclear

expression of SV40 which is then inconsistent with a transforming role and the low or null expression of SV40 antigens in many MM lines [40,41]. Clearly, this controversy is a major concern in public health, for millions of individuals in U.S. and western countries were potentially exposed to SV40 in poliovirus vaccines administered between 1955 and 1963 [4].

A major challenge to the diagnosis of mesothelioma is represented by the need to identify reliable prognostic factors in order to select for the best therapeutic option for different patients subgroup. Various prognostic variables have been considered so far, including symptoms (dyspnea, cough, chest pain), age, sex, histology, stage of disease and exposure to asbestos. Among these factors, histology is to have a major role: it has been identified, in fact, that mean survival for patients suffering from MM (whether untreated or treated with a single-modality therapy) is in the range of 4, 7 or 12 months depending on whether histology is sarcomatoid, biphasic or epithelioid. Histological presentation is also helpful in predicting mean survival after trimodal

therapy (extrapleural pleuropneumonectomy, chemotherapy and radiotherapy) [42]. It has been demonstrated, in fact, that post-treatment survival is strikingly dependent on the histotype, varying from 52% at two years in epithelioid MM to 16% at two years in sarcomatoid MM [43]. Another critical prognostic factor is tumor's primary site: patients suffering from peritoneal MM, in fact, are generally expected to have a median survival which is less than half of that with pleural MM [44]. A third important prognostic factor is disease staging: although mesothelioma is characterized by a low metastatic potential vs. local dissemination, the involvement of mediastinal lymph nodes or the presence of malignant cells in the effusions are both adverse prognostic factors [45].

The low overall survival rate reflects the most dramatic problem associated with MM: this neoplasm, in fact, is relatively resistant to conventional clinical approaches, including chemotherapy and radiation therapy. Single-drug chemotherapy is nowadays generally refused in MM

management, for partial response rates generally fall well below 20%, proving to be useless. To date, combinatory chemotherapy is preferred, with cisplatin + doxorubicin as an amenable protocol to obtain a 20 – 30% response. Similarly, radiation therapy or surgical treatment as single-modality treatments are relatively ineffective, although they can provide significant palliation [4]. Trimodal therapy (which is the association of surgical, chemotherapy and radiation therapy protocols) has proven to be highly successful in certain patients subgroups: eligible subjects suffering from pleural epithelioid MM, in fact, reach a significant 5-years survival after trimodal therapy. Unfortunately, only one-third of the subjects presenting pleural epithelioid MM are candidates to simultaneous surgical debulking of pleura, pericardium, diaphragm and invaded parts of lungs. Moreover, cytoreduction by massive surgical interventions is associated with a high mortality, and is considerably less effective in patients suffering from sarcomatoid MM [4,46]. Finally, since delays in MM diagnosis are frequent

mainly due to poor symptomatic signs, a great majority of MM patients are fairly sick at the time of diagnosis, rapidly lose weight and can't therefore candidate to trimodal therapy [4]. On these basis, novel therapeutic options for MM are highly needed, mainly on the pharmacological side of the problem. Promising approaches are constantly arising, with the exploitation of immune molecules or the engineering of immune cells as major upcoming examples [4,47].

## Tumor Immunology

Basing on Virchow's pioneer observations of leukocytes presence in solid tumors in 1863, in the 50's, the immunosurveillance (also called as immunological surveillance) hypothesis was formulated by Burnet. This is currently defined as a monitoring process of the immune system which detects and destroys newly arising neoplastic cells before they become a clinically evident tumor. In this view, an established tumor was seen as the dramatic result of a minor neoplastic subpopulation which somehow evaded efficient immune clearing [48,49]. Today, a huge amount of data challenge this hypothesis, leading to the paradoxical observation that the immune system may play key tumor-supporting roles, emphasizing that malignancies could grow because of spontaneous immune response [48,49]. It should be considered, in fact, that a dynamic ecological equilibrium establishes in a tumor focus: malignant cells threatening an organism challenge the immune system as much as the

immune system threatens neoplasm growth. By this way, each time the immune system clears a neoplastic growth, a positive pressure for the evolution of tumor itself is provided, so that a secondary neoplasm will be less immunogenic than the first [50]. This is commonly referred to as “immunoescape”, a process in which malignant cells tend to reduce the expression of class-I MHC molecules as well as to loss or to reduce their antigen charge as much as possible to evade recognition and killing by the immune system. Breaking of the equilibrium occurs when a neoplastic clone is generated whose antigen charge is sufficiently low not to induce an effective immune response. However, the pressure-induced evolutionary mechanisms acting on a new neoplasm are not sufficient to explain why abundantly antigenic tumors (such as breast cancers and melanomas) are inefficiently killed by the immune system [48-51]. Moreover, they do not explain why tumor-infiltrating lymphocytes (TILs) are frequently found in neoplastic foci, without the tumor being controlled or rejected

[52,53].

Different cells from the innate and the acquired compartments of the immune response have been recalled to take part to immunological surveillance: among these, CD4+ T helper cells (Th) and CD8+ cytotoxic T lymphocytes (CTLs), $\gamma\delta$ T cells, natural killer cells (NK) as well as cells belonging to the monocyte/macrophage lineage have been widely studied [53,54]. The basic concept of CTLs' antitumoral activity relies on the idea that aberrant proteins (or oncogenic viral products) are expressed by tumor cells together with class-I MHC molecules. This would lead CTLs to form immunological synapses with target cells allowing functional transfer of oncolytic ezymes. Clearly, as neoplasm exposure of MHC molecules decreases, efficacy of CTLs response should decrease too. That's why a key role is to be played by Th cells (and maybe by $\gamma\delta$ T cells in specific body districts): tumor-restricted Th cells, in fact, could release immunostimulating molecules such as IL-2, TNF-$\alpha$ and IFN-$\gamma$ which would turn CTLs responses up. In the same way, Th

cytokines could activate NK cells. Unlike CTLs, these cells are activated by the absence of class-I MHC molecules on target cells, thus possibly clearing that cells which are positively selected by CTLs patrolling pressure [53,54]. NK activation by Th cytokines (such as IL-2 and IL-12) leads to lymphokine-activated killer cells (LAK), whose tumoricidal activity has been extensively confirmed in vitro [54]. Clearly, the low antigen-presenting potential of tumor cells would be a major barrier to immune clearing, unless cells from the monocyte/macrophage lineage differentiate to professional antigen-presenting cells (APCs) such as macrophages and dendritic cells. Tumoricidal activity of macrophages is efficiently observed in vitro [54,55], and this is likely to correspond to its contribute in vivo. As effector cells, in fact, macrophages can count on the release of lysosomal enzymes, the production of reactive oxidant intermediates and the release of tumoricidal molecules such as TNF-$\alpha$. Moreover, as true APCs, scavenging of tumor cell debris by phagocytosis allow macrophages to process target antigens, exposing

them to $CD8^+$ and $CD4^+$ cells via class-I and –II MHC molecules. Devoided of a direct tumoricidal activity, dendritic cells are best professional APCs. Capturing and processing antigens by phagocytosis, in fact, these cells can efficiently present them to lymphocytes by either class-I and –II MHC molecules. A higher MHC molecules expression in respect to macrophages, together with the presence of a wide array of costimulatory signals such as CD40, CD80, CD86 and CD28 and the ability to cross-prime CD4+ and CD8+ lymphocytes makes these cells the most active APCs in the periphery and the most important link between innate and adaptive immunity [53-55].

Although efficiently prepared to kill neoplasms in vitro, however, our immune system paradoxically fails in protecting us in vivo. Basing on discussed evidences, it is clear that immunoescaping is not the main mechanism of immune surveillance evasion in tumor foci. A plethora of immunosuppressive mechanisms, in fact, act as a network in tumor microenvironment to actively skew immune response far from its aim. This process, in which

immune cells and immune networks undergo critical reprogramming of their functions by a malignant offence is called “immunoediting”, and it is undoubtedly the main feature of tumor immunology [48-50].

A comprehensive view of all possible immunosuppressive mechanisms acting in tumor milieu is something far in the future, as most of them seem to critically depend on specific events which vary in dependence of what tumor and/or what immune cell is considered. To date, a quite puzzling scenario of down-regulated dendritic cell activities, reduced antigen recognition, loss of T cell function by down-regulation of the ζ chain of the T cell receptor (TCR), suppressed stimulation of CTLs, NK cells, B cells and development of specific immunosuppressive immune subpopulations such as regulatory T cells (Tregs), tolerogenic dendritic cells (tDCs), myeloid suppressor cells and tumor-associated macrophages (TAMS), is observed [56]. The mechanisms underlying all of these events have been only initially investigated so far, and new

evidences are constantly accumulating. Interestingly, it has been noticed that immunosuppressive factors in tumor milieu are often related to inflammation: in example, molecules such as prostaglandins (mainly of the E series) have been demonstrated to be a constant presence in tumor microenvironment, wherein they act as powerful suppressor of DCs, CTLs, NK, B cells and macrophages. Moreover, these molecules bias cytokine production, so that Th cells are skewed towards the production of Th2 cytokines such as TGF-β and IL-10. These, in turn, pleiotropically impair cell functions, leading to a generalized imbalance of immune responses. As an example, multiple deregulations in the production of oxidant molecules is frequently observed in phagocytic cells [56]. Perhaps, the newest evidences on an active association between tumors and immune cells come from the characterization of novel tumor-induced immunosuppressive cell types. These cytotypes seem to be minor subpopulations efficiently exploited by tumors, while their physiological roles remain unclear still.

Regulatory T cells (Tregs) are probably the best known immunosuppressive cells in both the maintenance of physiological self tolerance and tumor-induced peripheral immunosuppression. Tregs are a heterogeneous population of anergic $CD4^+$ T cells, generally described by the $Foxp3^+$ $CD25^+$ phenotype, whose activity is mainly directed towards the inhibition of both $CD4^+$ and $CD8^+$ cells. Among the various mechanisms proposed so far, the inhibition of IL-2 transcription, immunosuppression by the interplay between CTLA-4 on Tregs and CD80/86 on target T cells and the production of immunosuppressive cytokines such as IL-10 and TGF-$\beta$ seem to play major roles [57,58].

Tolerogenic dendritic cells (tDCs) are closely related to Tregs. Immature DCs, in fact, are thought to integrate different signals gathered from the surrounding immune environment, and to possibly develop to tDCs in the presence of immunosuppressive signals such as IL-10, TGF-$\beta$ and Tregs themselves [60]. These tDCs, in turn, could polarize naïve CD4+ Th towards Treg phenotype,

amplifying local immunosuppression in a “chain-reaction” way [59], even though the relative contribution of IL-10, TGF-β, indoleamine 2,3-dioxygenase (IDO) and more “exotic” GM-CSF, kynurenines, vasoactive intestinal peptide (VIP) and immunosuppressive exosomes is unclear at the moment [60-63].

Myeloid suppressor cells (MSCs) have been mainly identified by their tendency to accumulate in the bone marrow, blood, spleen and tumor sites of tumor-bearing individuals. MSCs still represent an open issue, for their generation has only been related to VEGF, GM-CSF and IL-1 so far, and their functions are puzzling [64-66]. To date, most of our knowledge about MSCs comes from in vitro studies, in which MSCs proved to be a complex group of IL-10, TGF-β and nitrite producers, with overwhelming of T cells by generation of tDCs, exhaustion of normal DC pool and direct inhibition of T cell functions by nitrite and reactive oxygen species as supposed mechanisms of action [64-66].

Tumor-associated macrophages (TAMs) are a

common feature in almost all malignant focus, often representing the majority of infiltrated leukocytes [67]. These cells are accounted to mostly derive from circulating monocytes recruited into the tumor mass by chemoattractant gradients (thus rising the question whether MSCs could represent their parental source [66,68]), although evidences have been provided for local macrophage proliferation. TAMs are nowadays considered as major representative of the so-called M2 phenotype, which is an alternative polarization of human macrophages in which downregulation of inflammatory activity is observed together with a rise in immunosuppressive activities [69]. Particularly, downregulation of inflammatory cytokines such as TNF-$\alpha$, IL-1, IL-12 and IFN-$\gamma$ has been variously observed, with a concomitant upregulation of IL-1 receptor antagonist and IL-1 decoy receptor, IL-10, $PGE_2$ and arginine metabolism shift towards the arginase pathway. Moreover, an upregulation in matrix-degrading activities is frequently observed, which is thought to reflect a tumor-prone mechanism facilitating

neoangiogenesis in malignant foci [67,69]. Coherently, a high number of TAMs is generally associated with a poor prognosis, as demonstrated by several studies on breast, lung, prostate, cervix, bladder and glial tumors, and by experimental evidences on malignant mesothelioma [67,70].

Finally, a growing interest is rising around somatic cell hybridization between cancer and immune cells. The concept of somatic hybridization is quite old in the literature, for it traces back to Aichel's theory of metastasis as the result of spontaneous cell fusion formulated in 1911 [71]. The basic idea of such a theory is that, since white blood cells are able to recognize and attack cancer cells, a possibility there is for joining of these cells to form a somatic hybrid. To date, many evidences have been proposed enlightening that, both *in vitro* and *in vivo*, cell fusion is an observable process [71-73]. Up to now, convincing data have been provided about somatic hybridization occurs between different cancer cells and macrophages, a cell type which is physiologically prone to fuse as a part of its development to an

osteoclast [71-73]. Clearly, hybrid cells are believed to exhibit a higher metastatic potential and possible new immunomodulatory functions, although main issues remain about both the “stability” of these hybrids (with a particular concern about the differences between etherokaryon – two nuclei coexisting in a single cytosol – and genomic hybrids – cells bearing a single nucleus in which chromosomes from different cells coexist) and their relative importance in *in vivo* cancerogenesis [71-73].

## Tumor Immunotherapy

Cancer immunotherapy is the use of the immune system to reject cancer, basing on the assumption that stimulating the patient's immune system could lead to the destruction of malignant tumor cells that are responsible for the disease. Areas of actual investigation include: vaccines, monoclonal antibodies, cytokines and other immunostimulants and adoptive transfer of effector cells and antigen presenting cells (APCs) [74].

Vaccination strategies are diversified, including both the use of tumor-specific antigens (specific gene products which are univocally expressed by malignant cells) and tumor-associated antigens (which are not univocally expressed by tumors but whose expression is highly increased in neoplasms) [75-78]. To date, several approaches have been attempted to accomplish effective vaccination: major strategies now include the use of autologous or allogeneic whole tumor cells or oncolysates, transfection of tumor cells with cytokine genes or

costimulatory molecules' genes, exploitation of tumor proteins and peptides, development of anti-idiotypic molecules and viral and bacterial vectors for gene therapy and tumor killing [74]. Each of these approaches has specific advantages and disadvantages, which actually limit their possible applications: in example, vaccination with whole tumor cells may fail in presenting sufficient antigen or costimulatory molecules to effector cells, while allogeneic neoplasms may lead to alloimmunity development [77,79,80]. Equally, tumor transfection may be ineffective on tumor masses in vivo and may induce detrimental systemic reactions as well [77-80]. Although attractive, tumor proteins and peptides are bound to disadvantages too, for they imply that tumor masses should clonally express the same epitopes without allelic variants, as well they are likely to induce a short-lasting response in the absence of "helper" peptides [81-83]. Likewise, anti-idiotypic molecules effectiveness is hampered by its own specificity, so that different tumor clones may be insensible to such molecules [74]. Finally, vectors

are burdened by disadvantages too, the decrease in vaccination efficacy at repeated immunization, the generation of protective host immunity against vector proteins and the transfection of variable amounts of exogenous, unwanted nucleic materials being major examples [74].

A more successful approach to clinical immunotherapy is the use of monoclonal antibody (mAb) therapy. More than two decades of clinical studies have now defined the burdens of mAbs therapy, either using unconjugated or conjugated Abs. Moreover, mAbs engineering strategies aiming at reducing immunogenicity or decreasing pharmacokinetic metabolism, as well as improving tumor penetrance and immunogenicity or enhancing cellular responses have been explored [74]. To date, mAb therapy is the foremost immunotherapy branch in clinical tumor management, with several mAbs being approved by the Food and Drug Administration (FDA) for use in humans: these include humanized mAbs such as Alemtuzumab for chronic lymphocytic leukemia, Bevacizumab and Panitumumab for

colorectal cancer, Gemtuzumab ozogamicin for acute myelogenous leukemia and Trastuzumab for breast cancer, as well as chimeric mAbs such as Cetuximab for colorectal cancer and Rituximab for non-Hodgkin lymphoma, and the murine mAb Ibritumomab tiuxetan for non-Hodgkin lymphoma [84-91]. Unfortunately, adverse side effects have been elucidated too: in example, anti-IgG immunity arises in patients administered with murine mAbs [74]. Moreover, both the Ab size and the dynamics of mAb penetrance following repeated administrations could obstacle Ab efficacy [74].

Another promising approach to tumor immunotherapy is represented by adoptive cell transfer. This can be achieved either by transferring APCs, such as DCs, which would in turn elicit immune responses or by transferring LAK, TILs, macrophages or antigen-specific lymphocytes [74,92]. APCs transfer is regarded to as the most promising tool for in vivo induction of antitumor responses, for these cells would be able to both process tumor antigens and to create de novo a robust immunostimulant

environment. A major concern in APCs usage, however, exists about the source of such cells (for it exists the possibility to obtain unwanted tDCs if using circulating monocytes from compromised patients) and the best strategy to load tumor epitopes inside DCs. Likewise, direct transfer of effector cells has disadvantages too, mainly regarding the ways lymphocytes are expanded ex vivo and the systemic toxicity of immunostimulant cytokines routinely infused together with these cells [74,93,94]. Recently, more successful results have been achieved by using tumor-restricted lymphocytes or T cells transfected in vitro with engineered T cell receptor (TCR). All of these approaches, however, are burdened by the disadvantage to face the established immunosuppressive tumor microenvironment when reinjected in the patients, a disadvantage which could clearly blunt the efficacy of any of these protocols [74,95].

The existence of immunosuppressive tumor microenvironment is the main rationale for another immunotherapy approach, which is cytokine therapy.

Consequently, cytokine-based immune intervention may be important for circumventing inhibitory mechanisms that disengage the development of a therapeutically relevant immune response. Generally, cytokines that act best on immune cells act subsequent to antigen recognition and cellular activation, since those processes are intimately needed to coordinate and govern cytokine receptor expression. On the basis of the spectrum and functional properties of distinct cytokines, IL-2, IL-12, IFN-$\gamma$ and GM-CSF have been associated with cell-mediated immunity and tumor regression [74]. Actually, cytokine therapy is regarded to as a strategy to ingrate other immunotherapy approaches, even though parameters such as dose, schedule and frequency of cytokine administration are complex and must be customized on the basis of their specific mode of action, pharmacokinetics and predicted time of utilization during the course of the developing immune response [74].

Finally, novel approaches have been developed that link immunotherapy to biotechnologies: among the

various proposal, the generation of hybrid cells obtained by the fusion of malignant cells with antigen-presenting cells are perhaps the most outstanding example [96-100].

## Aim

Malignant mesothelioma is a higly fatal tumor arising on body serousal membranes. Although issues are still pending about the carcinogenic mechanisms leading to the development of such a neoplasm, evidences have been accumulated for its significant immunomodulatory activity.

Despite this fact, however, many molecular mechanisms are still lacking to explain the interactions between mesothelioma and cells belonging to the monocyte/macrophage lineage. These cells, in fact, are likely to play a crucial role in mesothelioma pathogenesis, and their accumulation in mesothelioma foci is very likely to represent a negative prognostic factor.

Basing on these considerations, the aim of my research was to focus on the crosstalk between immune cells and malignant mesothelioma in order to enlighten hierarchical relationships driving both monocyte/macrophage deregulation and the establishment of tumor-supporting activities in

immune cells.

In this context, co-cultures and conditioned media have been used to allow interactions between malignant and immune cells, and data have been collected demonstrating different mechanisms determining tumor-induced physiopathological skewing of human monocytes and macrophages phenotypes and functions.

# Results and Discussion

## Part I - Differential effects of malignant mesothelioma cells on THP-1 monocytes and macrophages *

***Abstract***

Malignant mesothelioma (MM) is a highly fatal tumor arising from inner body membranes, whose extensive growth is facilitated by its week immunogenicity and by its ability to blunt the immune response which should arise from the huge mass of leukocytes typically infiltrating this tumor. It has been reported that the inflammatory infiltrate found in MM tissues is characterized by a high prevalence of macrophages. Thus, in this work we evaluated the ability of human MM cells to modulate the inflammatory phenotype of human THP-1 monocytes and macrophages, a widely used in vitro model of monocyte/macrophage differentiation. Furthermore, we tested the hypothesis that the exposure to MM cells could alter the differentiation of THP-1 monocytes favoring the development of alternatively activated, tumor-supporting

macrophages. Our data prove for the first time that MM cells can polarize monocytes towards an altered inflammatory phenotype and macrophages towards an immunosuppressive phenotype. Moreover we demonstrate that monocytes cocultivated with MM cells “keep a memory” of their encounter with the tumor which influences their differentiation to macrophages. On the whole, we provide evidence that MM cells exert distinct, cell-specific effects on monocytes and macrophages. The thorough characterization of such effects may be of a crucial importance for the rational design of new immunotherapeutic protocols.

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## *Materials and methods*

### *Cell Cultures*

The human monocytic cell line THP-1 [101] was obtained from the American Type Culture Collection (Manassas, VA, USA). The human pleural MM cell line Mero 84 [102] was a generous gift by Dr. Marjan Versnel. Both cell lines were cultured at 37°C in 5% $CO_2$ in DMEM supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 µg/ml streptomycin, 100 U/ml penicillin and 100 µg/ml sodium pyruvate (complete medium), all from Cambrex (Lonza Milano, Milan, Italy). THP-1 Mo were differentiated to MΦ by incubation with 100 nM phorbol 12-myristate 13-acetate (PMA; Sigma Aldrich, Milan, Italy) for 72 h in the dark, as previously described [103].

### *Experimental plan*

The effects of MM cells on the phenotype of THP-1 Mo were evaluated using the following experimental plan: THP-1 Mo were seeded in complete medium in

6-well plates at a density of 1 to $3x10^5$ cells/well; each well was then covered with a cell culture insert (pore size: 0,4 μM; Sigma Aldrich) containing an equal amount of Mero 84 cells seeded in the same medium (THP-1/Mero 84 cell ratio 1:1); after 24, 48 or 72 h of cocultivation, the inserts were removed and the Mo were washed, counted and replated. The cultures containing Mero 84-exposed Mo (Mo-MM) and control cultures containing an equal number of Mo not exposed to Mero 84 cells were then stimulated with 5 μg/ml of LPS (Sigma-Aldrich) for 24 h and used for comparative phenotypic evaluations.

The effects of MM cells on the phenotype of THP-1 MΦ were evaluated using two different experimental settings: in the first setting, THP-1 Mo were differentiated to MΦ and then cocultivated with Mero 84 cells; in the second setting, Mo were first cocultivated with Mero 84 cells and then differentiated to MΦ. Cocultures were performed as described above, with a THP-1/Mero 84 cell ratio of 1:1. Equal numbers of MΦ, Mero 84-exposed MΦ (MΦ1-MM) and MΦ generated from Mero 84-

exposed Mo (MΦ2-MM) were then plated and stimulated with 5 μg/ml of LPS for 24 h. The cultures were finally used for comparative phenotypic evaluations as described below.

The growth and survival rate of THP-1 Mo and MΦ cultures were routinely assessed by cell counts using the trypan blue exclusion method; proliferation and survival of THP-1 Mo and MΦ were found to be unaffected by the cocultivation with MM cells and by LPS treatment (data not shown).

***[$^3$H]-Arachidonic acid release assay***

THP-1 Mo were labelled for 3 h with 1 μCi [$^3$H]-arachidonic acid ([$^3$H]-AA, specific activity 202,4 Ci/mmol; PerkinElmer Italia, Monza, Italy) at 37°C in serum-free medium, as previously described [104]. The labelled cells were then washed twice in serum-free medium to remove unincorporated [$^3$H]-AA and used to obtain replicate cultures of labelled Mo, MΦ, Mo-MM, MΦ1-MM and MΦ2-MM. The cultures were finally stimulated with LPS for 24 h. Cultures containing [$^3$H]-AA-labelled, LPS-unstimulated Mo

and MΦ were used to control basal [$^3$H]-AA release. Culture media were collected and 100 µl aliquots were added to 3 ml Optifluor (PerkinElmer Italia) and analyzed by a liquid scintillator counter.

***Measurement of Prostaglandin $E_2$, Tumor Necrosis Factor-$\alpha$ and Interleukin-10 release***

The amounts of Prostaglandin $E_2$ ($PGE_2$), Tumor Necrosis factor-$\alpha$ (TNF-$\alpha$) and Interleukin-10 (IL-10) released in the culture media by LPS-stimulated Mo, MΦ, Mo-MM, MΦ1-MM and MΦ2-MM and by LPS-unstimulated Mo and MΦ, were quantified using a monoclonal $PGE_2$ EIA kit (Cayman Chemicals, Italy Cabru, Arcore, Italy), an ELISA kit for TNF-$\alpha$ and an ELISA kit for IL-10 (both from Pierce Endogen, Celbio, Milan, Italy) according to manufacturers instructions.

***Evaluation of nitrite production by Griess reaction***

The amount of nitrite ($NO_2$) released in the culture media of LPS-stimulated Mo, MΦ, Mo-MM, MΦ1-MM and MΦ2-MM and of LPS-unstimulated Mo and MΦ was quantified as previously described [105].

Briefly, equal volumes of culture media and Griess modified reagent (Sigma-Aldrich) were mixed in a macrotitre plate. Upon incubation for 30 min at room temperature, the presence of $NO_2$ in the samples determines the formation of a fluorescent product sensible to stimulation with a laser beam set at 550 nm. $NO_2$ concentration in each sample was thus determined by plotting the sample O.D. at 550 nm on a standard curve prepared with sodium nitrite solutions (concentration range: 0,43 – 65 µM).

***Phagocytosis assay***

The phagocytic activity of THP-1 cells was evaluated by measuring the uptake of FITC-dextran particles by flow cytometry, as previously described [106]. In brief, THP-1 Mo, MΦ, Mo-MM, MΦ1-MM and MΦ2-MM were stimulated with LPS in the presence of 1 mg/ml FITC-Dextran (MW: 70 KDa; Sigma-Aldrich) for 24 h in the dark. Control Mo and MΦ were also incubated with FITC-Dextran in the absence of LPS, in order to assess basal phagocytic activity. At the end of incubation, the cells were extensively washed,

recovered by centrifugation and analyzed by FACS.

***Collagenolytic activity assay***

The serum-free culture media of LPS-stimulated Mo, MΦ, Mo-MM, MΦ1-MM and MΦ2-MM and of LPS-unstimulated Mo and MΦ were collected and ten-fold concentrated using Centricon devices (cut-off: 10 KDa; Millipore, Milan, Italy). Aliquots (200 μl) of concentrated media were mixed with 100 μl of fluorescein-labelled collagen (final concentration: 2,5 mg/ml; Sigma-Aldrich) and incubated for 2 h in the dark at 37°C. Each mixture was brought to a final volume of 2 ml with phosphate-buffered saline (PBS) and the sample fluorescence was analyzed using a computer-aided background-controlling fluorimeter (Perkin Elmer LS 50 b), with the excitation wavelength set at 485 nm, the emission wavelength set at 530 nm, and slits respectively set at 5 and 10 nm for each light pathway [107].

***Statistical analysis***

Data distribution was preliminarily verified by the

Kolmogorov-Smirnov test, and data sets were analyzed by one-way analysis of variance (ANOVA) followed by Newman-Keuls test. Differences were regarded as significant when p value was less than 0,05. Quantitative data were expressed as the percentile mean ± S.D. of at least four replicate determinations, each performed in triplicate, in respect to control levels arbitrary set to 100, except where otherwise indicated.

***Results***

***Effects of MM cells on the release of [$^3$H]-AA by THP-1 Mo and MΦ***

The release of AA from membrane phospholipids is an important marker of Mo and MΦ activation [108-110]. Therefore, in order to characterize the possible immunomodulatory effects exerted by MM cells on the THP-1 lineage, we first evaluated whether the cocultivation with Mero 84 cells could affect the release of [$^3$H]-AA by LPS-stimulated THP-1 Mo and MΦ.

As illustrated in Fig. 1A, LPS-stimulated Mo-MM released less [$^3$H]-AA than LPS-stimulated, non-cocultivated Mo. In particular, the amounts of [$^3$H]-AA released by LPS-stimulated Mo-MM generated by coculturing THP-1 Mo with Mero 84 cells for 24, 48 or 72 h were similar to those released by unstimulated Mo, and about 25% lower than those of LPS-stimulated Mo. Conversely, as illustrated in Fig. 1B, MΦ1-MM obtained by coculturing MΦ with MM cells for 24-72 h released significantly more [$^3$H]-

AA than non-cocultivated MΦ in response to LPS. Moreover, also the LPS-stimulated MΦ2-MM generated from Mo exposed to MM cells for 24-72 h released higher amounts of [$^3$H]-AA as compared to LPS-stimulated, non-cocultivated MΦ.

Since the effects observed after 24-72 h of cocultivation were similar, a 24 h cocultivation time was chosen for all subsequent experiments.

***Effects of MM cells on $PGE_2$ release by THP-1 Mo and MΦ***

$PGE_2$ is one of the major AA metabolites and it is known to play immunosuppressive functions in the tumor microenvironment [35,111]. Thus, it was evaluated whether the cocultivation with Mero 84 cells could modulate the release of $PGE_2$ by LPS-stimulated THP-1 Mo and MΦ.

Consistent with the results obtained in [$^3$H]-AA release studies, the cocultivation with MM cells had opposite effects on the release of $PGE_2$ by THP-1 Mo and MΦ. Indeed, as shown in Fig. 2A-B, LPS-stimulated Mo-MM released lower amounts of $PGE_2$

as compared to non-cocultivated, LPS-stimulated Mo, while the amounts of $PGE_2$ released upon LPS stimulation by both MΦ1-MM and MΦ2-MM were higher than those released by non-cocultivated MΦ.

***Differential effects of MM cells on TNF-α and IL-10 release***

To further evaluate the ability of MM cells to modulate the phenotype of THP-1 cells, experiments were conducted to assess whether the coculture with Mero 84 cells could affect the release of the prototypical pro-inflammatory cytokine TNF-α and the prototypical anti-inflammatory cytokine IL-10 [64] by THP-1 Mo and MΦ. Data in Fig. 3A show that the release of TNF-α induced by LPS was about ten-fold lower in Mo-MM *vs.* the non-cocultivated Mo, while the amounts of IL-10 released in response to LPS by Mo and Mo-MM were similar. Conversely, data in Fig. 3B show that non-cocultivated MΦ, MΦ1-MM and MΦ2-MM released comparable amounts of TNF-α upon LPS stimulation, whereas both MΦ1-MM and MΦ2-MM showed higher levels

of LPS-induced IL-10 release than non-cocultivated MΦ.

***Effects of MM cells on $NO_2$ production***

According to different authors, the polarization towards an M2 phenotype is accompanied by a reduction in the capacity to produce NO [112-114]. In order to assess whether the cocultivation with MM cells could affect the production of NO by THP-1 Mo and MΦ, the concentration of $NO_2$ in culture media was measured as a surrogate of NO production [105,115]. Indeed $NO_2$ is the major metabolite of NO and its extracellular concentration is directly proportional to that of NO [105].

Data in Fig. 4A-B show that upon LPS stimulation Mo and Mo-MM produced similar amounts of $NO_2$. On the other hand, both MΦ1-MM and MΦ2-MM produced lower levels of $NO_2$ than non-cocultivated MΦ.

***Effects of MM cells on Mo and MΦ phagocytic activity***

Next, we focused on the effects of MM cells on the phagocytic properties of THP-1 Mo and MΦ. Data in Fig. 5A show that LPS-stimulated Mo-MM displayed a small increase in the ability to phagocytize FITC-dextran particles as compared to non-cocultivated, LPS-stimulated Mo. However, as illustrated in fig. 5C this increase did not reach statistical significance. On the other hand, as illustrated in Fig. 5B-D, the phagocytic activity of both MΦ1-MM and MΦ2-MM was significantly lower than that of non-cocultivated MΦ upon LPS-stimulation, being almost equal to that of unstimulated, non-cocultivated MΦ.

***MM cells up-regulate the collagenolytic activity of both THP-1 Mo and MΦ***

Finally, it was assessed whether the cocultivation with MM cells could modulate collagen degradation by THP-1 Mo and MΦ. Interestingly, MM cells exerted comparable effects on the collagenolytic activity of the two cell types. Indeed, as illustrated in

Fig. 6A-B, the collagenolytic activity of LPS-stimulated Mo-MM was nearly doubled as compared to that of the non-cocultivated, LPS-stimulated Mo and, similarly, the collagenolytic activity of LPS-stimulated MΦ1-MM and MΦ2-MM was more than doubled as compared to that of the non-cocultivated, LPS-stimulated MΦ.

The reported effects exerted by MM cells on the phenotype of THP-1 Mo and MΦ are summarized in Table I.

**Discussion**

This paper represents the first direct evidence of the broad immunomodulatory effects exerted by pleural MM cells on Mo and MΦ. Mononuclear phagocytes are among the earliest immune cells infiltrating tumor sites and they nominally take part to almost all the phases of the anti-tumor reaction [50]. In fact, tumor-infiltrating Mo can effectively switch on the inflammatory reaction by releasing chemoattractant and immunostimulating factors and can differentiate into MΦ, thus empowering the innate response while bridging to the acquired response [50]. On the other hand, tumors can develop several strategies to counteract immune effector cells and escape recognition and destruction by the immune system. For instance, many of the soluble factors occurring in the cancer microenvironment are able to suppress the anti-tumor functions of Mo and MΦ or even to alter Mo/MΦ functional programs, thus transforming these cells into powerful tumor's allies [50,116].

In the attempt to shed light upon the immunomodulatory activities of MM, we developed

a cocultivation framework to evaluate the effects of MM cells on the phenotype of both Mo and MΦ of the THP-1 lineage. Furthermore, we evaluated whether MM cells could polarize Mo development into immunosuppressive MΦ.

The first evidence we provide for the immunomodulatory properties of MM cells is represented by their effects on the release of [$^3$H]-AA by THP-1 Mo and MΦ. Indeed our data demonstrate that the cocultivation with MM cells blunts the release of [$^3$H]-AA by Mo in response to an inflammatory stimulus, while it conversely polarizes MΦ to over-release [$^3$H]-AA in response to the same stimulus. Following its release, most AA is metabolized to a large family of pro- and anti-inflammatory metabolites in a cell-specific manner [111,117]. When the LPS-induced release of $PGE_2$, a major AA metabolite with anti-inflammatory properties [111,118] was assessed, it resulted that after the cocultivation with MM cells the amount of $PGE_2$ released by Mo was significantly reduced while, at the opposite, MΦ displayed a significant up-

regulation of $PGE_2$ release. Interestingly, it has been reported that MM tissues show high levels of $PGE_2$ [119], and that $PGE_2$ production in tumor sites can promote the development of regulatory T cells (Tregs), which in turn dramatically hamper the effectiveness of the anti-tumor immune response [120].

A further evidence of the differential immunomodulatory effect exerted by MM on the Mo/MΦ lineage is represented by the cytokine profile expressed by THP-1 Mo and MΦ after exposure to MM cells. In fact, following cocultivation with MM cells, the LPS-induced release of the prototypic M1 cytokine TNF-$\alpha$ [64] was markedly decreased in Mo cultures, while it appeared to be unaffected in MΦ cultures. Conversely, the cocultivation with MM cells enhanced the release of the prototypic M2 cytokine IL-10 [64] by LPS-stimulated MΦ, but did not modify the amount of IL-10 released by LPS-stimulated Mo. Moreover, as assessed through the measurement of $NO_2$, the LPS-stimulated release of NO, which is one of the most

important tumoricidal molecules produced by the Mo/MΦ lineage [113], was not affected in Mo cocultivated with MM cells, whereas it was significantly reduced in MM-exposed MΦ. Collectively, these data indicate that MM cells alter the phenotypic properties of Mo, while more clearly shifting MΦ towards an M2-like, protumoral phenotype [64,66,68]. In fact, the increased production of the immunosuppressive $PGE_2$ and IL-10 and the decrease of NO release observed in MM-exposed MΦ are both consistent with a shift towards the acquisition of M2 features [64,112,114,118]. Still, this MΦ polarization towards an immunosuppressive phenotype was not associated with a reduction in the release of TNF-α. Even though TNF-α is considered as a prototypical M1 cytokine, it also displays several tumor-supporting properties, including the ability to promote tumor growth, migration, invasion and angiogenesis [121]. Moreover, the production of such a molecule in developing MM foci is thought to mainly depend on MΦ and it has been linked to the malignant

transformation of mesothelial cells [34]. Thus, the observation that the LPS-induced release of TNF-α was not reduced in MM-exposed MΦ indicates that, while acquiring immunosuppressive properties, these cells maintain the expression of pro-inflammatory, tumor-supporting mediators.

We also observed that the cocultivation with MM cells had different effects on the phagocytosis of dextran particles by LPS-stimulated Mo and MΦ: the phagocytic activity of MM-exposed Mo was similar to that of non-cocultivated Mo, whereas the phagocytic activity of MM-exposed MΦ was significantly reduced as compared to that of non-cocultivated MΦ. In view of the results discussed so far, this finding is peculiar. Indeed, according to different authors, alternatively activated MΦ exhibit a poor antigen-presenting ability but a high capacity for phagocytosis and, accordingly, are efficient scavengers of apoptotic cells and cell debris [112,122]. On the other hand, an inefficient clearance of apoptotic cells can elicit pro-inflammatory as well as pro-tumoral,

immunosuppressive responses [35]. Therefore, the inhibition of the phagocytic activity observed in MM-exposed MΦ may play a double-edged role in the modulation of immune functions within the tumor microenvironment.

The observed ability of MM cells to decrease the release of TNF-$\alpha$ and $PGE_2$ without altering the production of IL-10 and $NO_2$ by THP-1 Mo indicates the instauration of an altered phenotype in Mo, which exhibit mixed pro- and anti-inflammatory features. It has been reported that tumor-associated Mo can exhibit immunosuppressive, M2-like properties. On the other hand, according to different authors, such Mo appear to display complex and multifaceted phenotypes and may develop from Mo undergoing a transient phase of activation followed by a phase of refractoriness to inflammatory stimuli [68,66,123,124]. The altered phenotype displayed by MM-exposed Mo may thus represent an intermediate stage toward the acquisition of more defined immunosuppressive properties.

Remarkably, as assessed by the release of lipid

mediators ([$^3$H]-AA, $PGE_2$), cytokines (TNF-$\alpha$, IL-10) and NO as well as by the phagocytic and collagenolytic activity, the phenotype of THP-1 MΦ cocultivated with MM cells (MΦ1-MM) was almost identical to that of MΦ generated by Mo cocultivated with MM cells (MΦ2-MM) for 24 h. This finding demonstrates for the first time that MM-exposed Mo are a "developmentally polarized" cell type, being yet committed to an altered differentiation after 24 h of cocultivation with MM cells. These Mo, in fact, differentiated into immunosuppressive MΦ in the absence of any further MM-derived factor added during the differentiation process, supporting the hypothesis that altered Mo are a privileged source of M2 MΦ.

Collagen degradation is crucially involved in the formation of new vessels as well as in tumor cell migration and invasion [125]. Unlike the differential effects discussed above, the cocultivation with MM cells induced a strong up-regulation of the collagen-degrading activity in both LPS-stimulated Mo and MΦ, demonstrating that either of these cell types

can be co-opted by the tumor as an effector of extracellular matrix degradation and tissue remodeling.

Taken together, our data demonstrate that MM polarizes Mo and MΦ towards distinct phenotypes and that that Mo “keep a memory” of their encounter with the tumor which influences their development to MΦ. By suggesting to target Mo in addition to MΦ [70] for the reversal of tumor-supporting immune cell phenotypes, our data may bear significance for the design of future immunotherapy approaches for MM.

The mechanisms by which tumor cells educate immune cells to exert tumor-supporting functions have not been fully elucidated to date [68]. The THP-1/MM cells coculture model could represent a valuable system to investigate the hierarchic role of MM-released factors involved in the modulation of the phenotype of Mo and MΦ.

# Figure 1

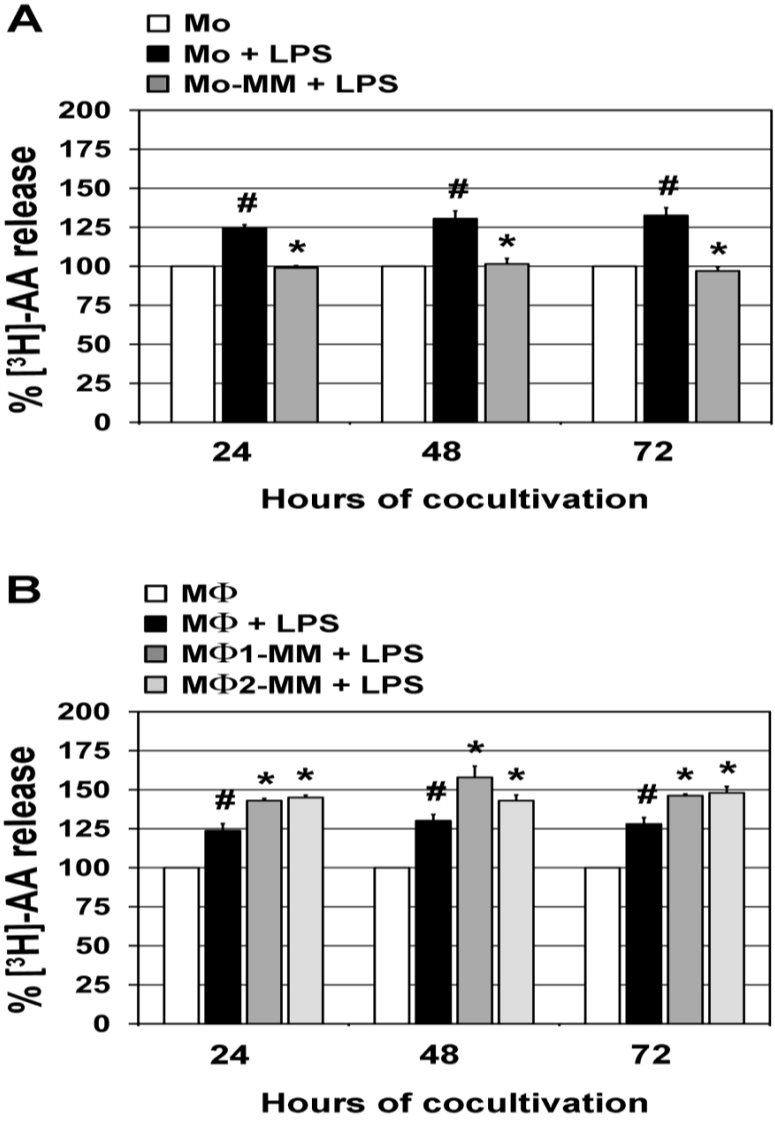
A
Mo
Mo + LPS
Mo-MM + LPS
% [3H]-AA release
200
175
150
125
100
75
50
25
0
#
*
24
48
72
Hours of cocultivation
B
MΦ
MΦ + LPS
MΦ1-MM + LPS
MΦ2-MM + LPS
% [3H]-AA release
Hours of cocultivation

**Figure 1. Effects of MM cells on the release of [$^3$H]-AA by THP-1 Mo and MΦ**.

(A) Percentile mean ± S.D. of [$^3$H]-AA release by unstimulated Mo, LPS-stimulated Mo and LPS-stimulated Mo-MM obtained by coculturing Mo with MM cells for 24, 48 and 72 h. (B) Percentile mean ± S.D. of [$^3$H]-AA release by unstimulated MΦ, LPS-stimulated MΦ, LPS-stimulated MΦ1-MM obtained by coculturing MΦ with MM cells for 24-72 h, and LPS-stimulated MΦ2-MM generated by Mo cocultured with MM cells for 24-72 h and then differentiated to MΦ. #$p < 0.05$ *vs.* unstimulated Mo or MΦ and *$p < 0.05$ *vs.* LPS-stimulated Mo or MΦ, as evaluated by ANOVA followed by Newman-Keuls test.

## Figure 2

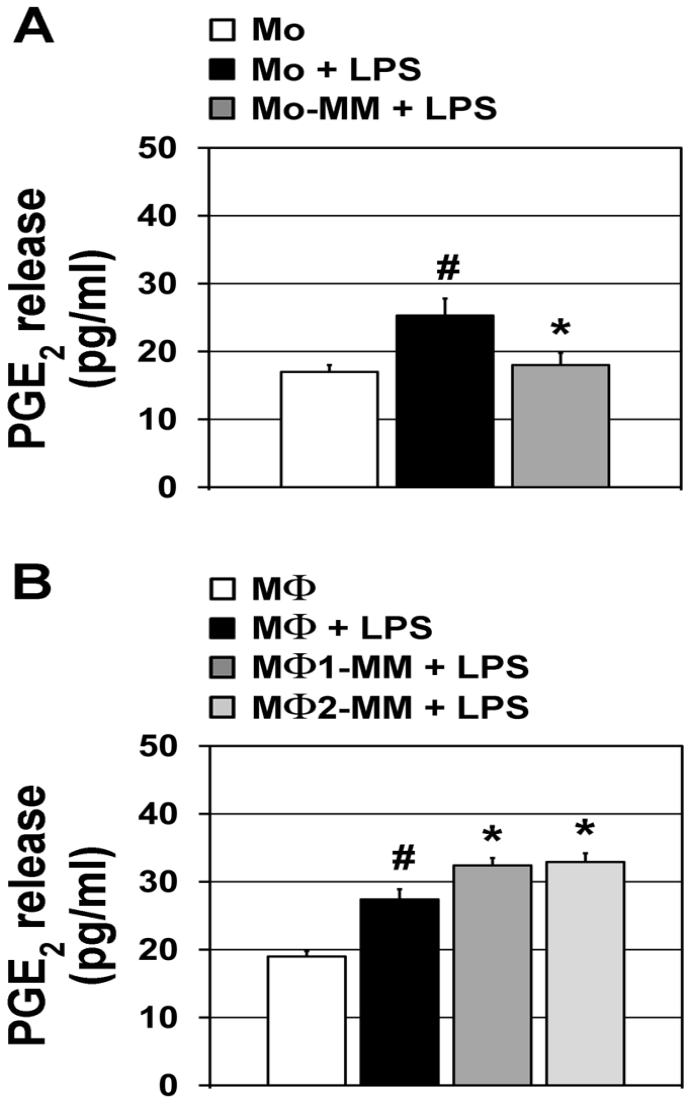
A
Mo
Mo + LPS
Mo-MM + LPS
PGE2 release (pg/ml)
50
40
30
20
10
0
#
*
B
MΦ
MΦ + LPS
MΦ1-MM + LPS
MΦ2-MM + LPS
PGE2 release (pg/ml)
50
40
30
20
10
0
#
*
*

**Figure 2. Effects of MM cells on $PGE_2$ release by THP-1 Mo and MΦ.**

(A) Mean ± S.D. values of $PGE_2$ release (pg/ml) by unstimulated Mo, LPS-stimulated Mo and LPS-stimulated Mo-MM obtained by coculturing Mo with MM cells for 24 h. (B) Mean ± S.D. values of $PGE_2$ release (pg/ml) by unstimulated MΦ, LPS-stimulated MΦ, LPS-stimulated MΦ1-MM obtained by coculturing MΦ with MM cells for 24 h, and LPS-stimulated MΦ2-MM generated by Mo cocultured with MM cells for 24 h and then differentiated to MΦ. #$p < 0.05$ *vs.* unstimulated Mo or MΦ and *$p < 0.05$ *vs.* LPS-stimulated Mo or MΦ, as evaluated by ANOVA followed by Newman-Keuls test.

# Figure 3

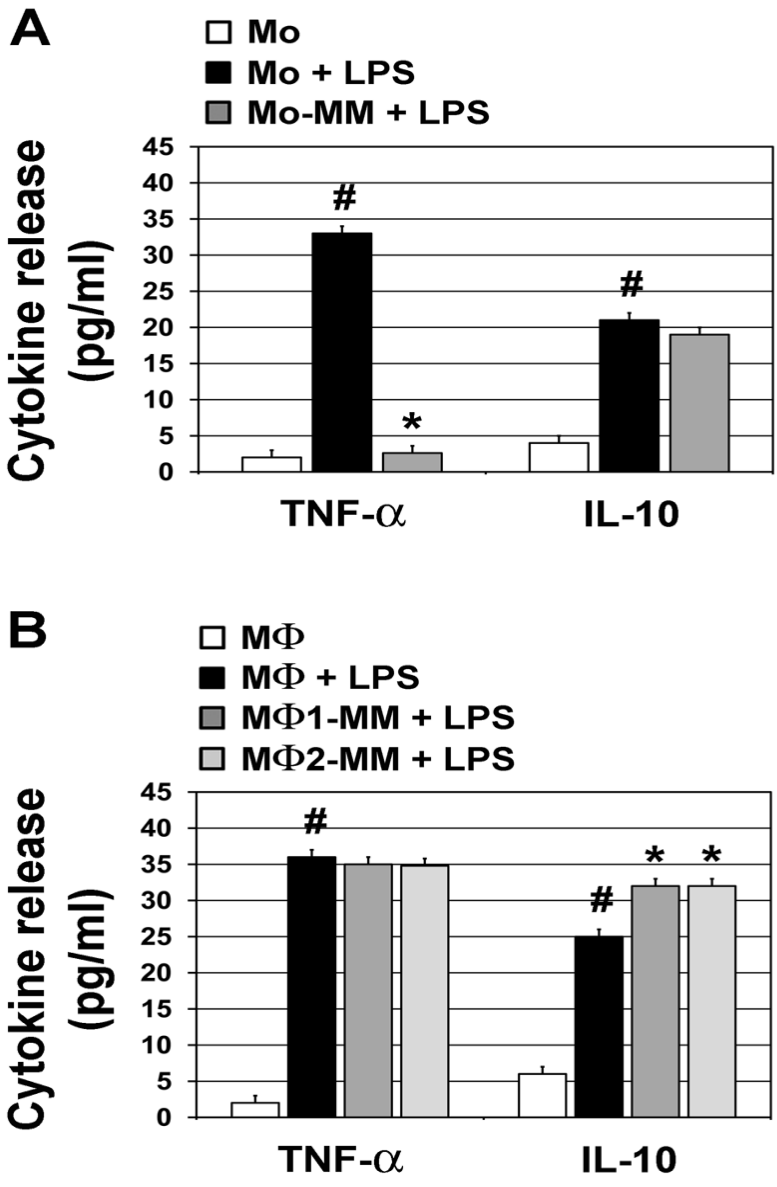
A
Mo
Mo + LPS
Mo-MM + LPS
Cytokine release (pg/ml)
45
40
35
30
25
20
15
10
5
0
#
*
#
TNF-α
IL-10
B
MΦ
MΦ + LPS
MΦ1-MM + LPS
MΦ2-MM + LPS
Cytokine release (pg/ml)
45
40
35
30
25
20
15
10
5
0
#
#
*
*
TNF-α
IL-10

**Figure 3. Effects of MM cells on TNF-α and IL-10 release.**

(A) Mean ± S.D. values of TNF-α and IL-10 release (pg/ml) by unstimulated Mo, LPS-stimulated Mo and LPS-stimulated Mo-MM obtained by coculturing Mo with MM cells for 24 h. (B) Mean ± S.D. values of TNF-α and IL-10 release (pg/ml) by unstimulated MΦ, LPS-stimulated MΦ, LPS-stimulated MΦ1-MM obtained by coculturing MΦ with MM cells for 24 h, and LPS-stimulated MΦ2-MM generated by Mo cocultured with MM cells for 24 h and then differentiated to MΦ. #$p < 0.05$ *vs.* unstimulated Mo or MΦ and *$p < 0.05$ *vs.* LPS-stimulated Mo or MΦ, as evaluated by ANOVA followed by Newman-Keuls test.

# Figure 4

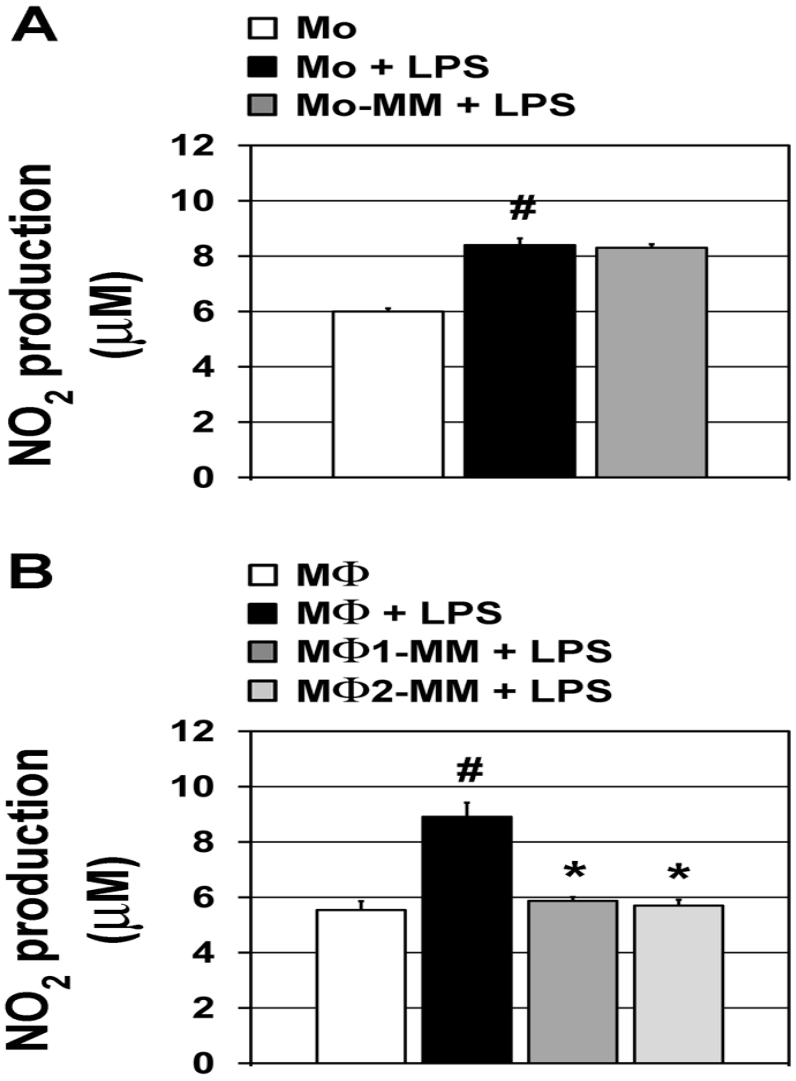
A
Mo
Mo + LPS
Mo-MM + LPS
NO2 production (μM)
12
10
8
6
4
2
0
#
B
MΦ
MΦ + LPS
MΦ1-MM + LPS
MΦ2-MM + LPS
NO2 production (μM)
12
10
8
6
4
2
0
#
*
*

**Figure 4. Effects of MM cells on $NO_2$ production**

(A) Mean ± S.D. values of $NO_2$ (μM) produced by unstimulated Mo, LPS-stimulated Mo and LPS-stimulated Mo-MM obtained by coculturing Mo with MM cells for 24 h. (B) Mean ± S.D. values of $NO_2$ (μM) produced by unstimulated MΦ, LPS-stimulated MΦ, LPS-stimulated MΦ1-MM obtained by coculturing MΦ with MM cells for 24 h, and LPS-stimulated MΦ2-MM generated by Mo cocultured with MM cells for 24 h and then differentiated to MΦ. #$p < 0.05$ *vs.* unstimulated Mo or MΦ and *$p < 0.05$ *vs.* LPS-stimulated Mo or MΦ, as evaluated by ANOVA followed by Newman-Keuls test.

# Figure 5

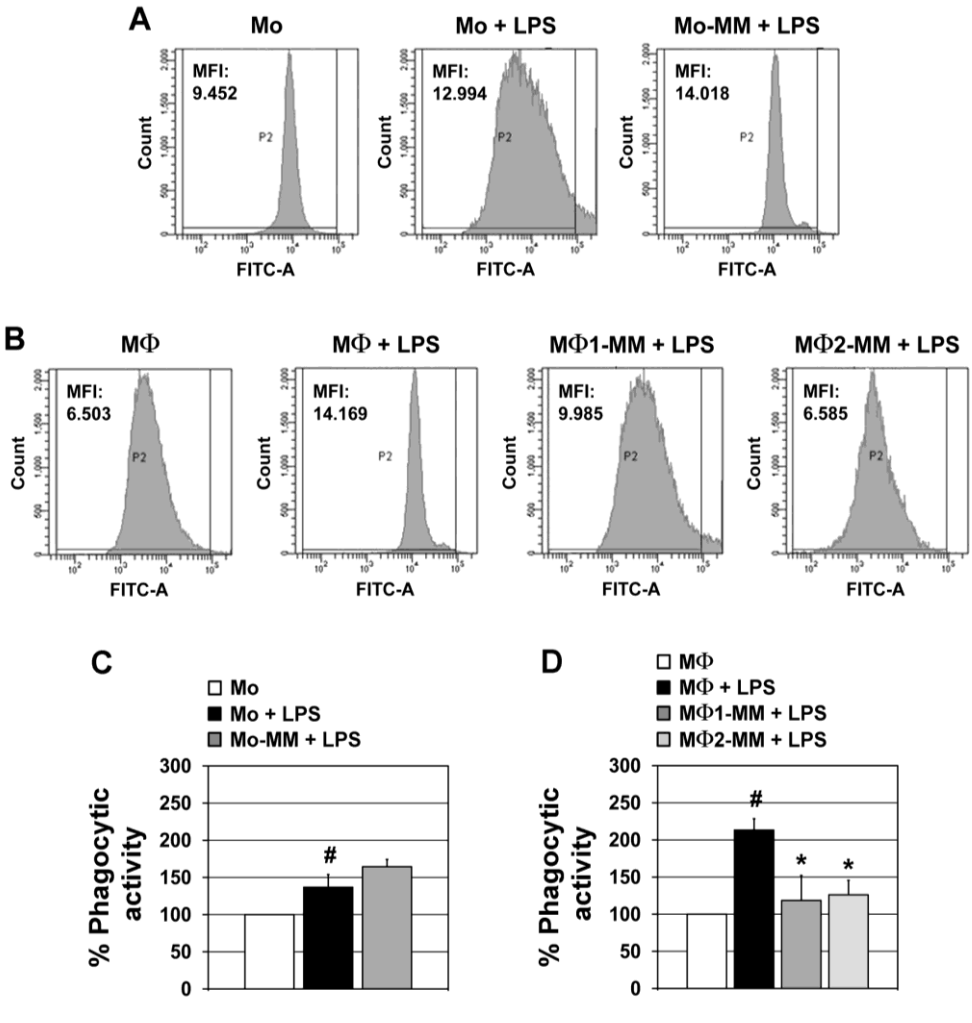
A
Mo
Mo + LPS
Mo-MM + LPS
MFI: 9.452
MFI: 12.994
MFI: 14.018
P2
Count
FITC-A
B
MΦ
MΦ + LPS
MΦ1-MM + LPS
MΦ2-MM + LPS
MFI: 6.503
MFI: 14.169
MFI: 9.985
MFI: 6.585
C
Mo
Mo + LPS
Mo-MM + LPS
% Phagocytic activity
300
250
200
150
100
50
0
#
D
MΦ
MΦ + LPS
MΦ1-MM + LPS
MΦ2-MM + LPS
#
*
*

**Figure 5. Effects of MM cells on Mo and MΦ phagocytic activity as assessed by flow cytometric analysis of FITC-dextran uptake.**

(A) A representative determination of FITC-dextran uptake by unstimulated Mo, LPS-stimulated Mo and LPS-stimulated Mo-MM obtained by coculturing Mo with MM cells for 24 h; the mean fluorescence intensity (MFI) is indicated in each case. (B) A representative determination of FITC-dextran uptake by unstimulated MΦ, LPS-stimulated MΦ, LPS-stimulated MΦ1-MM obtained by coculturing MΦ with MM cells for 24 h, and LPS-stimulated MΦ2-MM generated by Mo cocultured with MM cells for 24 h and then differentiated to MΦ; the MFI is indicated in each case. (C) Percentile mean ± S.D. of the phagocytic activity of unstimulated Mo, LPS-stimulated Mo and LPS-stimulated Mo-MM as determined from four different FITC-dextran uptake experiments. (D) Percentile mean ± S.D. of the phagocytic activity of unstimulated MΦ, LPS-stimulated MΦ, LPS-stimulated MΦ1-MM and MΦ2-MM as determined from four different FITC-dextran

uptake experiments. #p < 0.05 *vs.* unstimulated Mo or MΦ and *p < 0.05 *vs.* LPS-stimulated Mo or MΦ, as evaluated by ANOVA followed by Newman-Keuls test.

**Figure 6**

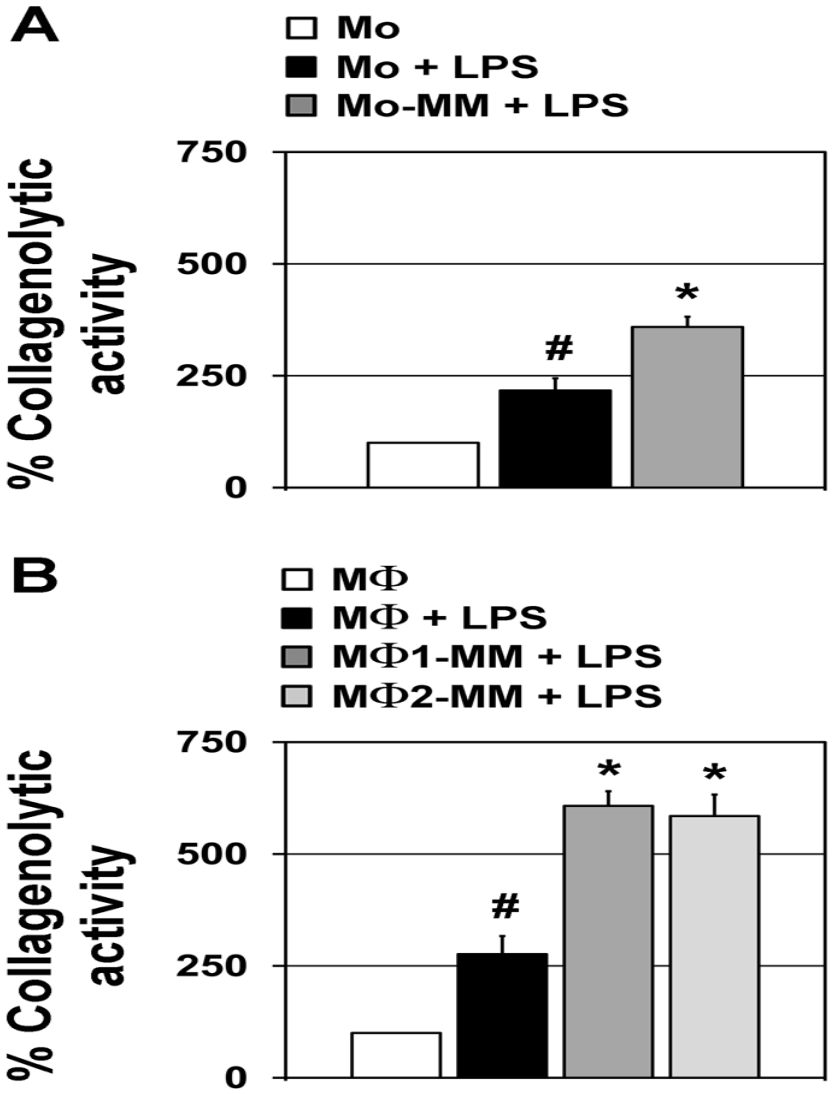
A
Mo
Mo + LPS
Mo-MM + LPS
% Collagenolytic activity
750
500
250
0
#
*
B
MΦ
MΦ + LPS
MΦ1-MM + LPS
MΦ2-MM + LPS
% Collagenolytic activity
750
500
250
0
#
*
*

**Figure 6. MM cells up-regulate the collagenolytic activity of THP-1 Mo and MΦ.**

(A) Percentile mean ± S.D. of the collagenolytic activity of unstimulated Mo, LPS-stimulated Mo and LPS-stimulated Mo-MM obtained by coculturing Mo with MM cells for 24 h. (B) Percentile mean ± S.D. of the collagenolytic activity of unstimulated MΦ, LPS-stimulated MΦ, LPS-stimulated MΦ1-MM obtained by coculturing MΦ with MM cells for 24 h, and LPS-stimulated MΦ2-MM generated by Mo cocultured with MM cells for 24 h and then differentiated to MΦ. #$p < 0.05$ *vs.* unstimulated Mo or MΦ and *$p < 0.05$ *vs.* LPS-stimulated Mo or MΦ, as evaluated by ANOVA followed by Newman-Keuls test.

**Table I. Differential effects of MM cells on the phenotype of LPS-stimulated THP-1 Mo and MΦ.**

| | Mo-MM vs. Mo | MΦ1-MM vs. MΦ | MΦ2-MM vs. MΦ |
|---|---|---|---|
| AA release | - | + | + |
| $PGE_2$ release | - | + | + |
| TNF-α release | - | ns | ns |
| IL-10 release | ns | + | + |
| $NO_2$ production | ns | - | - |
| Phagocytic activity | ns | - | - |
| Collagenolytic activity | + | + | + |

+: significantly increased; -: significantly decreased; ns: not significantly modified

**Part II – Interleukin-17 is produced by malignant mesothelioma-conditioned immune cells and promotes tumor growth and invasiveness ***

***Abstract***

Malignant mesothelioma (MM) is a fatal tumor of inner body membranes, whose extensive growth depends on both weak immunogenicity and the ability to reprogram surrounding immune cells towards tumor-supporting phenotypes. Interleukin-17 (IL-17) is a major inflammatory cytokine which is now accepted as the paradigmatic cytokine of many autoimmune diseases. However, its role in tumor immunology has only been partially unrevealed at the moment, and no data exist about its possible effects on malignant mesotheliomas. Thus, we evaluated both the ability of MM to induce the production of IL-17 by MM-conditioned immune cells and the effects of IL-17 on MM cell lines growth and invasiveness. Our data show, for the first time, that monocytes and macrophages are polarized by MM-

conditioned medium to produce IL-17, and that such cytokine exerts different tumor-supporting effects on both cell growth and invasiveness. These data could provide the evidence of a novel feedback mechanism between MM and immune cells and could also suggest potential targets for the development of new pharmacological protocols for MM treatment.

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## Materials and methods

### *Cell cultures and conditioned media.*

Mero-84 cell line was a generous gift of Dr. Marjan Versnel. Mesothelioma cell lines employed in this work belonged to the epithelioid (Mero-84 and H-Meso-1), the biphasic (MM-B1) and the sarcomatous (MM-F1) histotype. All cell lines were cultured at 37°C in 5% $CO_2$ in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 μg/ml streptomycin, 100 U/ml penicillin. Since we previously demonstrated the ability of Mero-84 cells to differentially affect monocyte/macrophage phenotype [126], the media from all MM cell lines were used to evaluate immune cells polarization. Conditioned medium was prepared by starving routinely-growing MM cells in serum-free medium for 24 h and then seeding them in serum-free medium in T 75 Falcon flasks until confluence. Medium aliquots were then collected and subjected to three consecutive centrifugations (300 x *g* for 10 minutes, 2000 x *g* for 20 minutes and 10000 x *g* for 30 minutes) to eliminate floating cells and debris.

***Assessment of IL-6 and IL-17 production by MM cells.***

$1x10^5$ MM cells were seeded in 6-well plates and incubated overnight in complete medium. The cells were then washed twice in PBS and incubated in serum-free medium supplemented with 0,2% bovine serum albumin (BSA), in the presence or absence of IL-17 (10 – 100 ng/ml) . Two μg/ml brefeldine A was added to all the wells to prevent exocytosis. The cells were then collected and stained with a PE-conjugated anti-IL-6 or anti-IL-17 mAb using specific buffers and following manufacturer's instructions. Data were acquired using a FACSCalibur and analyzed using CellQuest software.

***Immunophenotypical analysis of immune cells.***

Freshly isolated PBMCs, monocytes or macrophages were pre-incubated with MM-derived medium for 24 hours and then stimulated with 1 μM phorbol 12-myristate 13-acetate (PMA) and 10 μM ionomycin for 6 hours, in order to allow cytokines synthesis. Release of cytokines was inhibited by adding 1 μg/ml

brefeldine A 4 hours before the end of stimulation. At the end of the incubation, cells were washed twice and stained with specific surface antibodies in PBS, supplemented with 0.5% FBS and 0.02% $NaN_3$. Cells were fixed with 4% paraformaldehyde for 10 min at room temperature. Cells were stained intracellulary with anti-TNFα-APC, anti-TGF-β-PE, anti-IL-6-APC or anti-IL17-PE mAbs and analysed on FACSCalibur running CellQuest software.

***Survival assay and propidium iodide (PI) assay.***

In order to evaluate possible IL-17 effects on MM cells, 3 x $10^3$ cells/well were seeded in 6-well plates, allowed to adhere overnight and then starved in medium devoid of serum and containing 0,2% BSA with 10 or 100 ng/ml of IL-17. The plates were then incubated for 96 h at 37°C, and finally washed twice in PBS and stained with Giemsa. Micrographs were acquired using an inverted light microscope and image analysis was performed using ImageJ software.

To further evaluate IL-17 effects on MM cell growth,

plates were prepared as reported above and cells were collected in ice-cold PBS following 96 h incubation. The cells were then stained with 5 μg/ml propidium iodide (PI) to discriminate living (*dim*) and dead (*bright*) cells. Data were acquired using a FACSCalibur and analyzed using CellQuest software.

***Sulforhodamine B (SRB) assay.***

3 x $10^3$ MM cells were seeded in 96-well plates allowed to adhere overnight and incubated for 96 h in serum-free medium supplemented with 0,2% BSA, plus or not IL-17 (10 – 100 ng/ml). When requested, soluble interleukin-6 receptor (sIL-6R, 10 ng/ml) was added alone or together with IL-17 in the same medium as above. At the end of incubation, SRB assay was performed following manufacturer's instructions. The optical density (OD) of SRB-stained cultures was measured by an automated spectophotometric plate reader at a wavelength of 490 nm. To evaluate the possible interaction between sIL-6R and IL-17, R-indexes were calculated by multiplying the percentile survival of cells

incubated with different IL-17 concentrations by the percentile survival cells incubated with sIL-6R alone, and dividing the product by the percentile survival of cells treated with IL-17 (10 or 100 ng/ml) plus sIL-6R. A value greater than 1 roughly indicates a synergic activity.

***Carboxyfluorescein succinimidyl ester (CFSE) assay.***

$1x10^5$ MM cells were seeded in 6-well plates and incubated overnight in complete medium. The cells were then washed twice in PBS and stained with 1 μM carboxyfluorescein succinimidyl ester (CFSE) for 30 min in the dark The cells were then washed twice in PBS and incubated for 96 h in RPMI medium supplemented with 0,2% BSA in the presence or absence of IL-17 (10 – 100 ng/ml). Cells were then recollected, fixed in 4% paraformaldehyde and analyzed for fluorescence in FACScalibur running CellQuest software.

***PCR analysis for vascular endothelium growth factor (VEGF), placenta growth factor (PLGF), VEGF receptors and Neuropilins mRNAs.***

Total RNA was extracted from cells with Trizol according to the manufacturer's instructions. One µg of total RNA was reverse-transcribed using 200U of Superscript III, 10mM DTT, 20U of RNAse inhibitor, 2.5µM of random hexamers, 1mM of each dNTP in a final volume of 20 µl. Reactions were performed at 42°C for 60 min. The cDNA product (1µl) was amplified with 1.5U of Platinum Taq DNA polymerase in a final volume of 50 µl containing 200 µM of each dNTP and 50 pmol of each primer. The sequences of the primers were as previously reported [127]. Amplification consisted of 30 sec at 94°C, 30 sec at 65°C and 30 sec at 72°C for 35 cycles, preceded by a first step of 2 min at 94°C to allow activation of the enzyme and followed by a final extension at 72°C for 7 min. Primers were designed to span over different exons in order to avoid amplification of contaminating genomic DNA. Moreover, primers for VEGF and PlGF were designed to detect each of the

described alternative splicing isoforms. The identity of the amplicons was confirmed by sequencing.

***Wound healing assay.***

2,5 x $10^5$ MM cells were plated in 6-well plates in complete medium and allowed to adhere overnight. The cells were then washed and incubated for 24 h in serum-free medium supplemented with 0,2% BSA and IL-17 (10 – 100 ng/ml). When requested, 10 μg/ml Mitomycin C was added 30 min before the addition of 100 ng/ml IL-17 to inhibit cell proliferation. The plates were then washed twice in PBS, stained with Giemsa and analyzed under inverted light microscope. Image analysis was performed using ImageJ software.

***Statistical analysis.***

Data distribution was preliminarily verified by the Kolmogorov-Smirnov test, and data sets were analyzed by one-way analysis of variance (ANOVA) followed by Newman-Keuls test. Differences were regarded to as significant when *p*-value was < 0,05.

Quantitative data were expressed as the mean ± S.D. of at least three replicated determinations, except where otherwise indicated.

***Materials***

RPMI 1640 medium, FBS, L-glutamine, streptomycin and penicillin were from Euroclone. BSA, brefeldine A, PMA, ionomycin, paraformaldehyde, PI, SRB, sIL-6R and CFSE were from Sigma. All mAbs and specific staining buffers were from eBioscience. Trizol, Superscript III, DTT, RNAse inhibitor, random hexamers, dNTPs and Platinum Taq DNA polymerase were from Invitrogen.

**Results**

**Assessment of IL-17 production by MM and MM-associated immune cells.**

Although the cytokine profile of MM cells has been deeply investigated so far [3], no evidences can still be found about the production of IL-17 by MM cells and/or by the immune cells infiltrating MM foci, including monocytes (Mo), macrophages (Mϕ) and T cells. Thus, we started evaluating the basal production of IL-17 by the three different MM histotypes. Data in Fig. 1a show that all tested MM cell lines stained negative (*dim*) for the production of IL-17 after both 24 and 48 h of cultivation, evidencing that IL-17 is not produced by MM cells under standard conditions. Conversely, we show that both Mo and Mϕ. underwent a significant polarization towards an IL-17–producing phenotype when cultivated for 24 h in different MM-conditioned media, as shown by data in Fig. 1b, while $CD4^+$ and $CD8^+$ T cells did not, as evidenced by data in Fig. 1c, evidencing that MM effects on IL-17 polarization are mainly directed towards the monocyte/macrophage

lineage.

**IL-17 induces significant growth of MM cells.**

Since recent evidences have been provided about the ability of IL-17 to actively support the growth of different malignancies [128-130], experiments were performed to determine whether IL-17 could exert an effect on the growth of different MM cell lines. At first, survival assays were performed in order to evaluate the behavior of such a cytokine on cell growth. Data in Fig. 2a show that IL-17 induced a noteworthy survival increase at both 10 and 100 ng/ml in all cell lines in respect to control cells, supporting the hypothesis of a growth induction effect. To further confirm these data, survival assays were also performed by means of direct PI and SRB staining. Cytometrical data in Fig. 2b show that both tested IL-17 concentrations determined a significant decrease in the mean number of cells in the low right quadrant ($PI^{bright}$ cells), as expected by an increase in cell survival. High values observed in control cultures are clearly depending on the absence of serum in the

medium used for the experiments. A closely comparable situation was observed after SRB staining, as shown in Fig. 2c, which confirmed the significant increase in cell survival following incubation with IL-17.

Then, to finally confirm the proliferative activity of IL-17 on MM cells, experiments were performed using the intracellular dye CFSE. Since this dye is stably incorporated in the cytoplasm, its concentration halves as the cells undergo doubling so that cell fluorescence decreases as the cells proliferate. Data in Fig. 3 show that, at both 10 and 100 ng/ml, IL-17 induces a significant fluorescence decrease in all MM cell lines in respect to relative controls, confirming IL-17 ability to induce MM cell proliferation.

**Interleukin-6/Soluble Interleukin-6 receptor (IL-6/sIL-6R) axis is not involved in IL-17 – MM cell growth.**

IL-6 is one of the major cytokine being induced by IL-17 [131], and it is also a known mitogenic factor for MM cells [18]. However, its proliferative activity

could be blunted by the presence of defective IL-6 receptors (IL-6R) on cell membrane, so that it strongly relies on the release of soluble IL-6 receptor (sIL-6R) by different kinds of tumor-associated cells [132]. Having observed a significant growth increase in MM cells exposed to IL-17, we explored the possibilities that either IL-6 production could be induced in these cells by IL-17 or the addition of exogenous sIL-6R could exert any effect on IL-17-induced growth.

Data in Fig. 4a show that all MM cell lines did not produce IL-6 in response to IL-17, suggesting that such a cytokine is not involved in mediating IL-17 effects. Data in Fig. 4b confirmed the hypothesis, since it was shown that the addition of sIL-6R had no appreciable additive effects on IL-17 – exposed MM cells, as reported from R-indexes not significantly different from 1.

**Effects of MM-conditioned medium on Mo and Mϕphenotype.**

Basing on the above results, the cytokine profile of

MM-polarized Mo and Mϕ was further analyzed in order to explore its possible association with tumor-supporting functions. To this aim, cells were stained and analyzed for the production of those major cytokines known to synergize with IL-17, such as TNF-α [133], IL-6 [134] and TGF-β [135].

Fig. 5a shows the effects of MM-conditioned media on Mo. Data show that the medium from Mero-84 and MM-B1 determined an increase in IL-6 production, while the medium from H-Meso and MM-F1 decreased its production. Moreover, the medium from Mero-84, H-Meso and MM-B1 determined a remarkable increase in TGF-β expression, whereas the medium from MM-F1 had no effects. Finally, the medium from Mero-84 determined a noteworthy increase in TNF-α production while the medium from MM-B1 and MM-F1 appreciably decreased its production and the medium from H-Meso had no effects.

Fig. 5b shows the effects of MM-conditioned media on Mϕ. Data show that the medium from Mero-84, H-Meso and MM-B1 determined a significant

increase in IL-6 production, while the medium from MM-F1 had no effect. Moreover, the medium from Mero-84 and H-Meso significantly increased TGF-β production, while medium from MM-B1 didn't exert any effect and medium from MM-F1 induced a moderated decrease. Finally, the medium from Mero-84 and H-Meso respectively determined small increases in TNF-α production, while the medium from MM-B1 and MM-F1 showed no effect.

**IL-17 differentially affects the synthesis of VEGF, PlGF, VEGF receptors and Neuropilins mRNAs.**

Basing on recent evidences, a major effect of IL-17 is represented by its ability to stimulate the synthesis of different growth factors, such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) and hepatocyte growth factor (HGF) [136]. Moreover, IL-17 is involved in different trans-activation loops, which might further amplify its proliferative activity [133,137]. Thus, experiments were performed to evaluate whether IL-17 could modulate the synthesis of two known MM growth

factors, such as VEGF and PlGF [127], as well as their receptors VEGF-R1 and -R2 and their co-receptors neuropilin 1 and 2 (NP1 and NP2) that are involved in MM growth and survival [127,138].

Data in Fig. 6a show that IL-17 has a histotype-specific effect on both VEGF and PlGF: the cytokine, in fact, induced a significant VEGF mRNA upregulation in three out of four cell lines at both 10 and 100 ng/ml, while it was uneffective on MM-F1 at the lower dose. Differently, both the concentrations were able to induce PlGF mRNA upregulation in MM-B1 cells only, whereas they was clearly uneffective in both Mero-84 and H-Meso cells. Once again, IL-17 had a significant upregulatory effect on PlGF synthesis in MM-F1 cell only at the higher dose, though the lower concentration was uneffective.

Histotype-specific effects were also observed on VEGFRs and NPs mRNAs: whereas the expression of VEGF-R2 was unaffected at any dose in all cell lines, the expression of VEGF-R1 was upregulated by 100 ng/ml IL-17 in Mero-84, H-Meso and MM-B1 cells, but unaffected in MM-F1 cells. Moreover, significant

upregulation was also induced by 10 ng/ml IL-17 in Mero-84 cells. Similarly, NP1 mRNA was upregulated by both 10 and 100 ng/ml IL-17 in MM-B1 cells, whereas it was unaffected at each concentration in Mero-84, H-Meso and MM-F1. Conversely, significant upregulation of NP2 mRNA was observed in Mero-84 and H-Meso cells exposed to 100 ng/ml IL-17, while no noteworthy differences were observed at lower concentration on the same cells and at both doses in MM-B1 and MM-F1 cells. Results from densitometrical analysis are shown in Fig. 6b.

**IL-17 up-regulates MM cell invasiveness.**

Recent data have pointed out that IL-17 could promote cell invasiveness by different mechanisms, also including matrix metalloproteinases (MMPs) upregulation [130,139]. Basing on these evidences, wound healing experiments were performed to evaluate the possible effects of IL-17 on MM cell migration. Data in Fig. 7 show that 100 ng/ml IL-17 induced a considerable recovery of scratch area in all MM cell lines in respect to control cells, whereas 10

ng/ml IL-17 induced a significant scratch area recovery in Mero-84, H-Meso and MM-F1 cells but failed to induce a significant wound closure in MM-B1 cells. Moreover, to rule out the possible interference of IL-17 – induced cell growth on the wound healing process, experiments were replicated in the presence of mitomycin C, a cell replication inhibitor. Data in Fig. 7 show that no significant differences were observed when cells were incubated with 100 ng/ml IL-17 in the presence or absence of mytomicin, confirming that the cytokine induced a strong increase in cell migration, which is likely to reflect an increase in their invasive capability.

**Discussion**

Increasing evidences suggest that IL-17 may act a primary role at cancer sites, acting both on tumor and immune cells [128-130,139,140]. As far as the immune system is concerned, IL-17 is a prototypical pro-inflammatory cytokine, thus inducing the release of master inflammatory cytokine such as TNF-α, IL-1, IL-6 and many more [140,141]. Moreover, IL-17 has a strong effect on surrounding tissue cells, affecting the functions of both epithelial, endothelial and stromal fibroblasts [140]. Major uncertainty, however, resides still on what kind of cells are intratumoral producer of IL-17 as well as what the effects of such a cytokine are on different tumors. So far, IL-17 is regarded to as a typical $CD4^+$ T cell cytokine [140,141], although its production has also been observed in tumor-associated macrophages (TAMs) [139]. Coherently, IL-17 functions have crossed the burdens of inflammation, and evidences about its possible roles in tumors are continuously accumulating.

Notably, although malignant mesothelioma (MM) is a

neoplasia deriving from a cell type (the mesothelial cell) known to express IL-17 receptors [142], displaying both ephitelioid and sarcomatoid (fibrous) histotypes and a high leucocyte infiltrate, no data have been provided still for the evaluation of IL-17 effects on this tumor. Thus, aim of this work was to evaluate whether MM-associated immune cells could be able to produce such a cytokine, also evaluating its possible effects on MM cells.

Our data show for the first time that MM cells are surrounded by a favourable microenvironment which strongly polarizes monocytes (Mo) and macrophages (Mϕ) to produce IL-17, rising a phenotype which has been already observed in lung cancer [139]. Interestingly, such an observation strongly matches with our previous data, which supported the idea of a close interrelationship between MM and altered Mo/Mϕ phenotypes displaying major pro-tumoral activities [126]. Clearly, clarification of the cytokines participating to such a polarizing environment is still a major challenge. However, we ruled out the possibility of an IL-17 – driven loop since all MM lines

stained negative for such a cytokine. However, MM is known to produce many IL-17 – polarizing cytokines, such as IL-1, IL-6 and TGF-β [143], thus being possibly able to polarize immune cells without the need of IL-17 itself. The relationships occurring between Mo/Mϕ and MM cells was then further analyzed by evaluating the production of those cytokines known to empower or to synergize with IL-17, namely TNF-α[133], IL-6 [134] and TGF-β [135]. Interestingly, we observed puzzling results, which are likely to reflect histotype-specific effects: it could be noticed in fact that the epithelioid lines Mero-84 and H-Meso share a common trend in driving the production of the investigated cytokines from both Mo and Mϕ even if H-Meso decreased IL-6 production from Mo and didn't vary the production of TGF-β and TNF-α from Mϕ. Moreover, the biphasic line MM-B1 induced the production of IL-6 from both Mo and Mϕ and only increased TGF-β production from Mϕ, determining also a decrease in TNF-α production from Mo. Conversely, the sarcomatous line MM-F1 didn't drive any particular cytokines production either from Mo

or Mϕ, rather determining a decrease in IL-6 and TNF-α production from Mo and TGF-β from Mϕ. We then stated hypothesizing that IL-17$^{+}$ Mo/Mϕ could be MM allies, and that IL-17 could somehow favor tumor cells. Our data clearly evidenced that IL-17 exerted a strong proliferative effect on MM cells: direct microscopical observations as well as biochemical and cytofluorimetrical evaluations, in fact, demonstrated that the cytokine induced a significant increase in MM growth rates regardless of the histotype, with a positive side effect on cell viability, as observed by direct propidium iodine staining. These observations, thus, are coherent with recent findings on hepatocellular carcinoma, cervical tumors and breast cancer [128-130,139], and support the view of IL-17 as a tumor-promoting factor in several different type of cancers.

In order to get a deeper insight on the mechanism by which IL-17 induced such an effect, then, we started analyzing IL-17 effects on those growth factors which are known to be major modulators of MM proliferation.

Considering that IL-6 is an autocrine growth factor for MM cells [132] and that IL-6/IL-17 trans-signalling has already been observed in fibroblasts [134], we started evaluating its possible involvement in IL-17 – induced proliferation. Interestingly, we determined that IL-6 is not involved in mediating IL-17 effects. At first, in fact, we observed no significant up-regulation following IL-17 administration, evidencing a lack of IL-6 – driven responses in our cell lines. Furthermore, to rule out the possible lack of IL-6 effects due to the expression of aberrant IL-6 receptors [132], soluble IL-6 receptor (sIL-6R) was added together with IL-17, but still no significant differences were noticed in respect to MM cells exposed to IL-17 alone.

Conversely, a totally different scenario was observed when cells were probed for VEGF and PlGF production. All MM histotypes, in fact, underwent VEGF upregulation when incubated with IL-17. Moreover, histotype specificity was observed in cell sensitivity to the cytokine: it can be clearly observed, in fact, that 10 mg/ml IL-17 was the most effective dose in the epithelioid lines, while 100 ng/ml was the

only effective dose in the sarcomatous line (though very faint bands were observed). Coherently, both doses had almost the same effects on the biphasic cell line. Similarly, PlGF synthesis was unaffected by both IL-17 doses on the epithelioid lines, while it was increased by both the doses on biphasic and sarcomatous lines. Interestingly, basing on recent evidences, PlGF is likely to be the key factor in mediating IL-17 effects on cell survival [127], though further experiments are needed to evaluate the intracellular signaling pathways linking PlGF to IL-17. Finally, histotype-specific effects were also observed on VEGF/PlGF receptors (VEGF-R1 and -R2) and their co-receptors Neuropilin 1 and 2 (NP1 and NP2). We observed, in fact, that the epithelioid and the biphasic lines underwent significant VEGF-R1 upregulation when pulsed with IL-17, while the sarcomatous line did not respond in such terms to the cytokine. Moreover, only the epithelioid lines showed upregulation of NP2, while only the biphasic line upregulated NP1, and no line upregulated VEGF-R2. Collectively, these data account for a complex

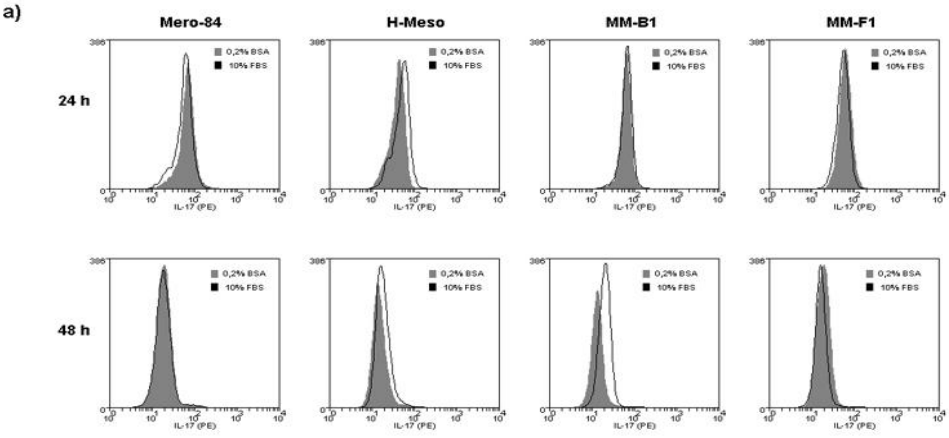
network of intracellular pathways regulating the effects of IL-17 on both VEGF, PlGF and their receptors, whose understanding will probably provide new tools to understand the mechanisms behind extensive MM growth.

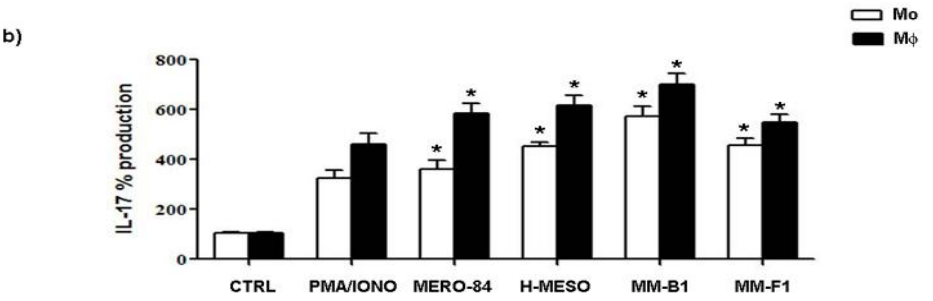
Finally, since IL-17 has been variously associated to an increase in cell invasiveness [130,133,139], MM cell mobility was tested in wound healing assays. Once again, we found that invasiveness was affected in a histotype-specific way in MM cells, so that the epithelioid lines dose-dependently responded to both IL-17 doses by increasing their invasiveness, while the biphasic and the sarcomatous cell line displayed higher invasiveness when exposed to the highest IL-17 dose. To ensure that such an effect was not dependent on cell proliferation, we also repeated the experiments adding Mytomicin C, to block the mitotic fuse and prevent proliferation [144]. Coherently, data showed that IL-17 effects on wound healing were not determined by cell proliferation, while they rather reside on an increase of the invasive capability of the cells. Basing on recent

evidences, a mechanism involving matrix metalloproteinases (MMPs) can be envisaged [139], though further experiments will be needed to unravel such a mechanism.

Taken together, our data prove for the first time a direct effect of MM cells on Mo/Mϕ, which are then polarized to produce IL-17. This cytokine, in turns, induces cell proliferation (probably via VEGF/PlGF upregulation) and augments invasiveness, thus strongly favoring MM cells. In the context of clarification of tumor - immune cells loops as a main goal for the development of novel and more active tumoricidal approaches, then, we propose our evidences as a novel finding whose further clarification could have a deep impact on MM chemo- and immunotherapy.

# Figure 1


a)
Mero-84
H-Meso
MM-B1
MM-F1
24 h
48 h
0,2% BSA
10% FBS
IL-17 (PE)


b)
Mo
Mϕ
IL-17 % production
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600
400
200
0
CTRL
PMA/IONO
MERO-84
H-MESO
MM-B1
MM-F1

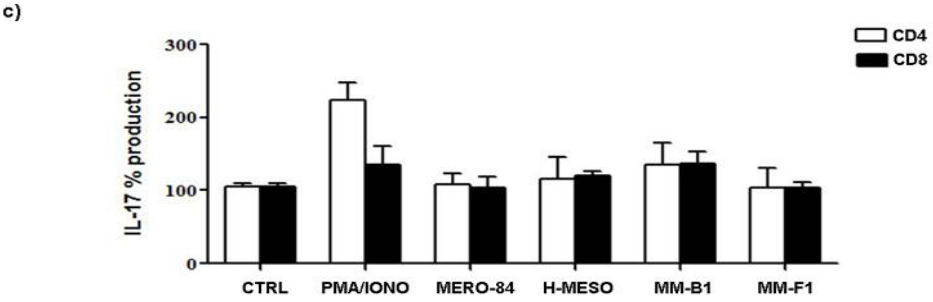
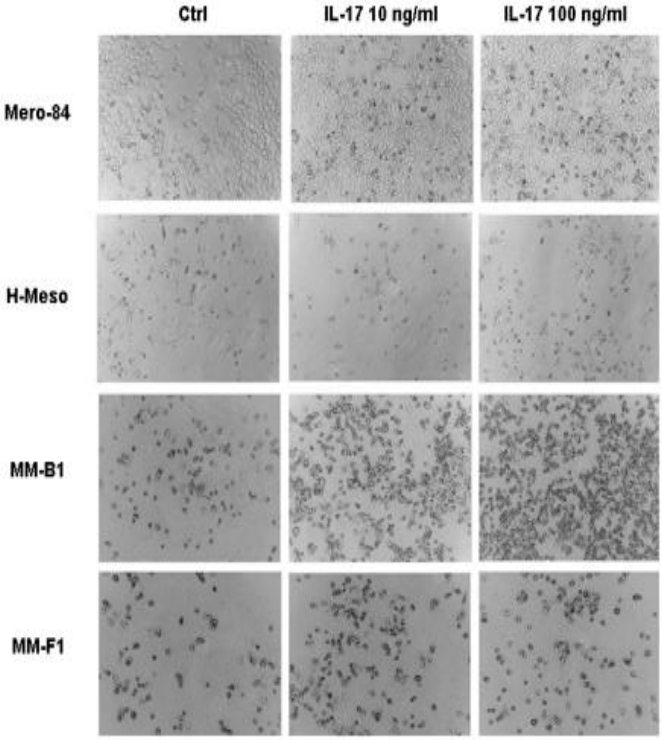

c)
CD4
CD8
IL-17 % production
300
200
100
0
CTRL
PMA/IONO
MERO-84
H-MESO
MM-B1
MM-F1

**Figure 1: IL-17 production by MM and MM-conditioned immune cells.**

a) 1 x $10^5$ MM cells were seeded both in serum free medium supplemented with 0,2% BSA or in complete medium (10% FBS) for 24 or 48 h and stained with PE-conjugated anti – IL-17 mAb. Data were acquired using a FACSCalibur running CellQuest software. A representative experiment is shown, which was repeated three additional times with similar results.

b) monocytes (Mo), macrophages (Mϕ) and c) T cells were obtained as reported in the Materials and Methods section, and incubated or not for 24 h in conditioned media mixed with fresh culture medium (1:1, V:V). Data were acquired using a FACSCalibur running CellQuest software, and expressed as percentile production in respect to unstimulated (Ctrl) cells arbitrary set to 100. *$p<0,05$, as from ANOVA followed by Newman-Keuls test, in respect to Ctrl cells.

## Figure 2a


Ctrl
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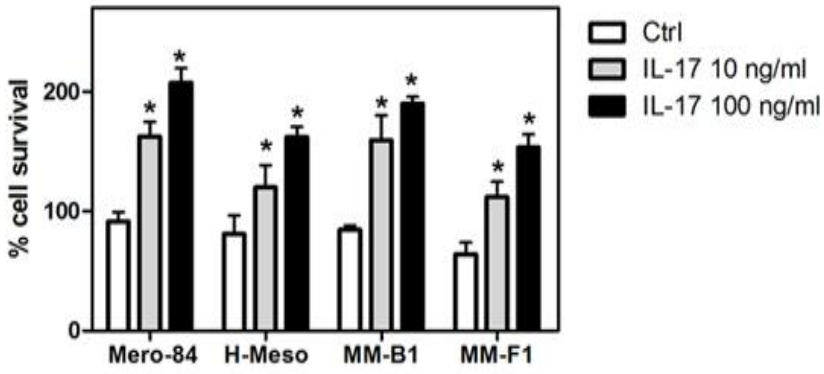
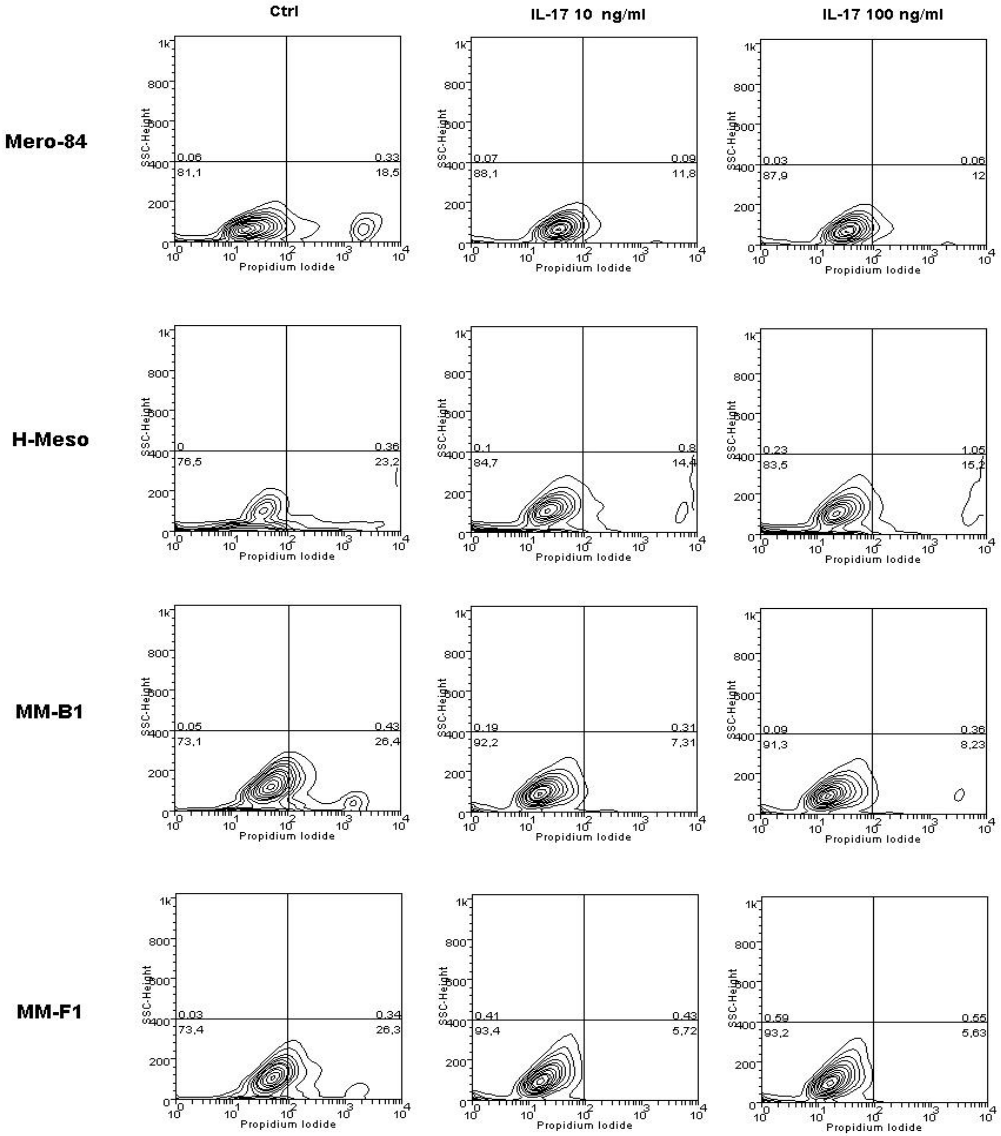
IL-17 100 ng/ml
Mero-84
H-Meso
MM-B1
MM-F1


% cell survival
200
100
0
Mero-84
H-Meso
MM-B1
MM-F1
Ctrl
IL-17 10 ng/ml
IL-17 100 ng/ml

# Figure 2b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IL-17 100 ng/ml
Mero-84
H-Meso
MM-B1
MM-F1
SSC-Height
Propidium Iodide
0.05
81,1
0.33
18,5
0.07
88,1
0.09
11,8
0.03
87,9
0.06
12
0
76,5
0.36
23,2
0.1
84,7
0.8
14,4
0.23
83,5
1.05
15,2
0.05
73,1
0.43
26,4
0.19
92,2
0.31
7,31
0.09
91,3
0.36
8,23
0.03
73,4
0.34
26,3
0.41
93,4
0.43
5,72
0.59
93,2
0.55
5,63

## Figure 2c

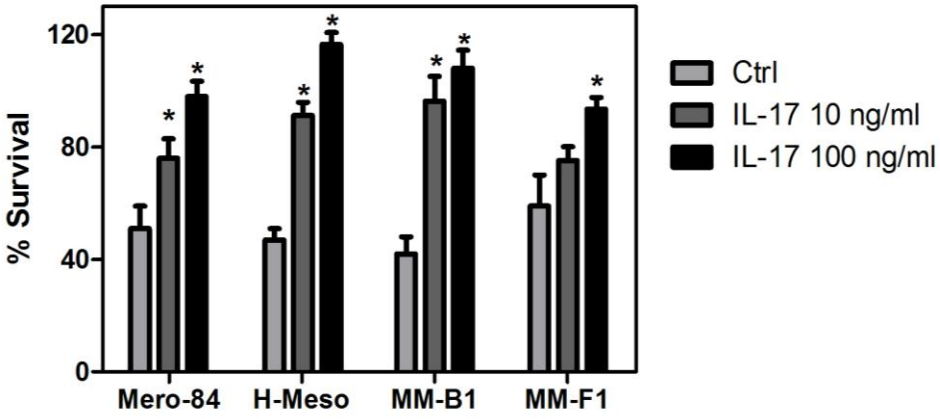

% Survival
120
80
40
0
Mero-84
H-Meso
MM-B1
MM-F1
Ctrl
IL-17 10 ng/ml
IL-17 100 ng/ml

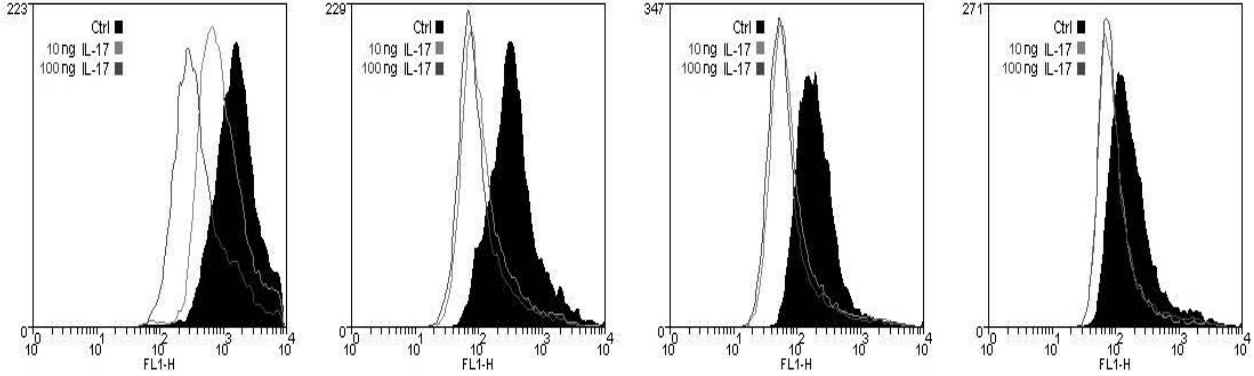
**Figure 2: Effects of IL-17 on the survival of MM cells.**

a) 3 x $10^3$ MM cells were seeded in 96-well plates and incubated for 96 h in serum-free medium supplemented with 0,2% BSA with or without different (10 or 100 ng/ml) IL-17 concentrations. The cells were then stained with Giemsa staining and analyzed under an inverted light microscope. Original magnification: 10 X. A representative experiment is shown, which was repeated three additional times with similar results. Percentile cell survival from all the experiments, calculated using ImageJ software, is shown below. *$p<0,05$, as from ANOVA followed by Newman-Keuls test, in respect to Ctrl (unstimulated) cells.

b) 3 x $10^3$ MM cells were seeded in 96-well plates and incubated for 96 h in serum-free medium supplemented with 0,2% BSA with or without different (10 or 100 ng/ml) IL-17 concentrations. The cells were then stained with propidium iodide and analyzed using FACSCalibur. A representative experiment is shown, which was repeated three additional times with similar results.

c) 3 x $10^3$ MM cells were seeded in 96-well plates and incubated for 96 h in serum-free medium supplemented with 0,2% BSA with or without different (10 or 100 ng/ml) IL-17 concentrations. The cells were then stained with SRB and analyzed for absorbance at 490 nm. Data are reported as the mean survival ± S.D. of four independent experiments. *p<0,05, as from ANOVA followed by Newman-Keuls test, in respect to Ctrl (unstimulated) cells.

# Figure 3




## MEAN FLUORESCENCE INTENSITY (MFI)

| Mero-84 | | | H-Meso | | | MM-B1 | | | MM-F1 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Ctrl | IL-17 10 ng/ml | IL-17 100 ng/ml | Ctrl | IL-17 10 ng/ml | IL-17 100 ng/ml | Ctrl | IL-17 10 ng/ml | IL-17 100 ng/ml | Ctrl | IL-17 10 ng/ml | IL-17 100 ng/ml |
| 1567,9 ± 21 | 850,5 ± 15 (*) | 358,7 ± 12 (*) | 333,8 ± 17 | 98,2 ± 14 (*) | 85,1 ± 13 (*) | 187,7 ± 24 | 66,1 ± 20 (*) | 59,4 ± 19 (*) | 156,8 ± 58 | 88,2 ± 13 (*) | 85,1 ± 18 (*) |

**Figure 3: Effects of IL-17 on the proliferation of MM cells.**

1 x $10^5$ MM cells were stained with 1 μM CFSE and incubated for 96 h in RPMI medium supplemented with 0,2% BSA with or without different (10 or 100 ng/ml) IL-17 concentrations. The cells were then fixed in 4% paraformaldehyde and analyzed for fluorescence using FACScalibur. A significant experiment is shown, which was repeated two additional times with similar results. Data in the table are reported as the mean fluorescence intensity (MFI) ± S.D. of three independent experiments. *p<0,05, as from ANOVA followed by Newman-Keuls test, in respect to Ctrl (unstimulated) cells.

# Figure 4

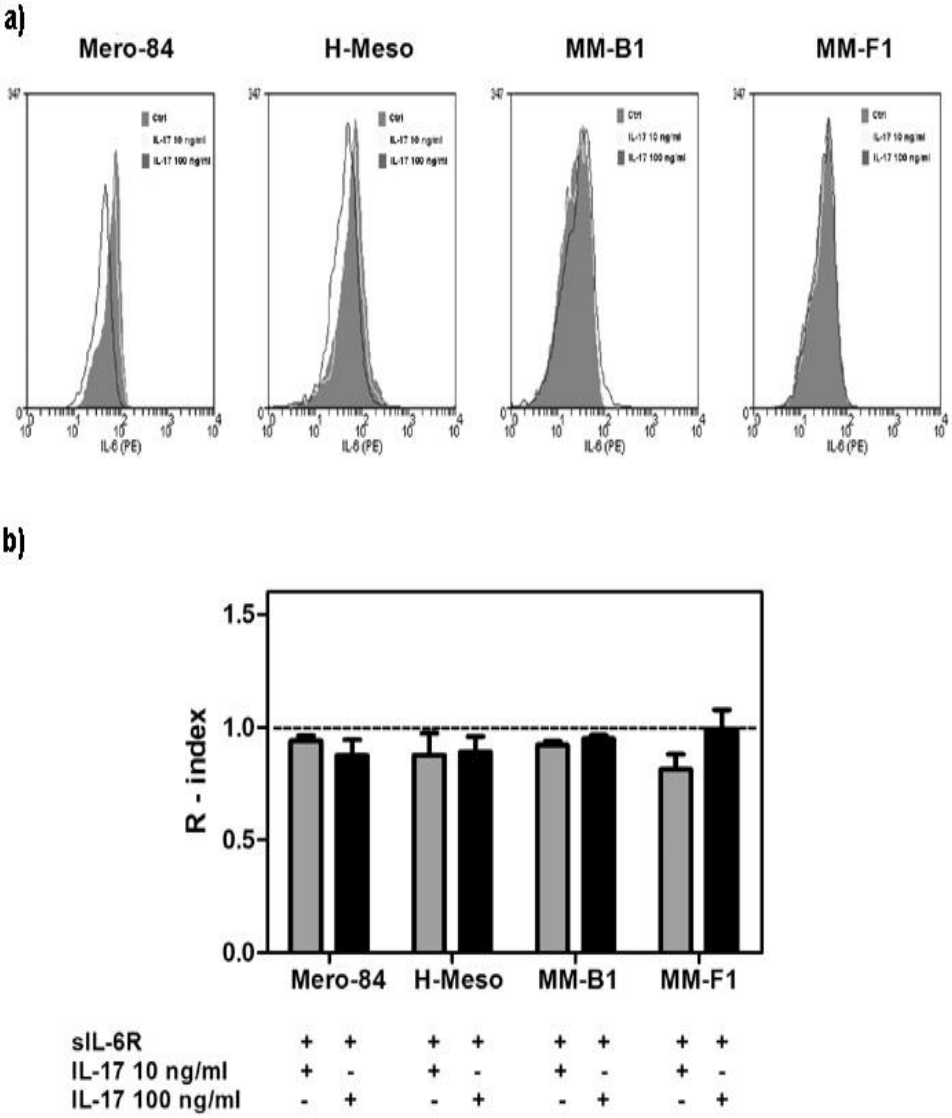

a)
Mero-84
H-Meso
MM-B1
MM-F1
Ctrl
IL-17 10 ng/ml
IL-17 100 ng/ml
IL-6 (PE)
b)
R - index
1.5
1.0
0.5
0.0
Mero-84
H-Meso
MM-B1
MM-F1
sIL-6R + + + + + + + +
IL-17 10 ng/ml + - + - + - + -
IL-17 100 ng/ml - + - + - + - +

**Figure 4: Effects of IL-6/sIL-6R on the proliferation of MM cells.**

a) 1,5 x $10^5$ MM cells were seeded in 6 well plates and incubated for 24 h in serum-free medium supplemented with 0,2% BSA with or without different (10 or 100 ng/ml) IL-17 concentrations. Cells were then fixed and stained for IL-6. A significant experiment is shown, which was repeated two additional times with similar results.

b) 3 x $10^3$ MM cells were seeded in 96-well plates and incubated for 96 h in serum-free medium supplemented with 0,2% BSA with or without 10 ng/ml sIL-6R and different (10 or 100 ng/ml) IL-17 concentrations. The cells were then stained with SRB and analyzed for absorbance at 490 nm. Data are shown as R-index, calculated as reported in the Materials and Methods section.

**Figure 5**

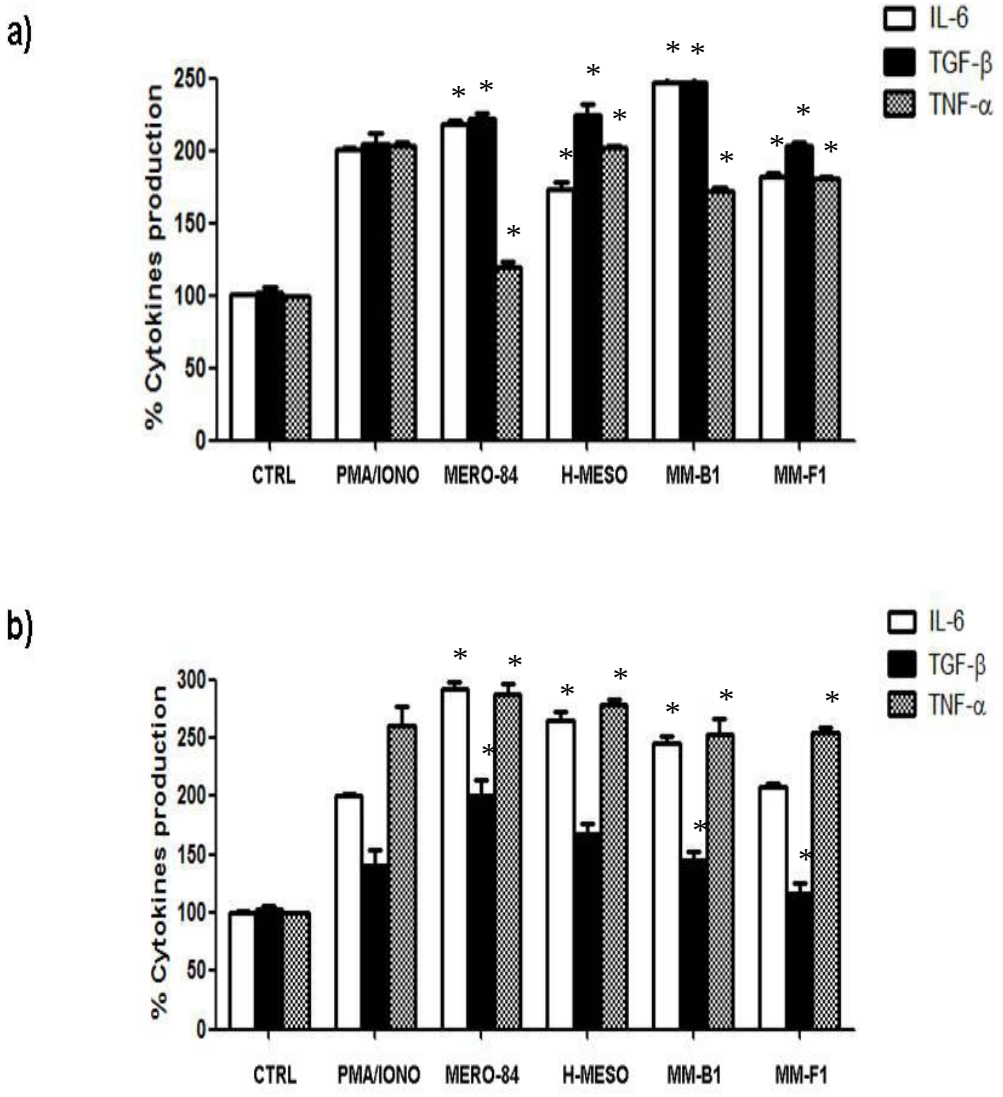
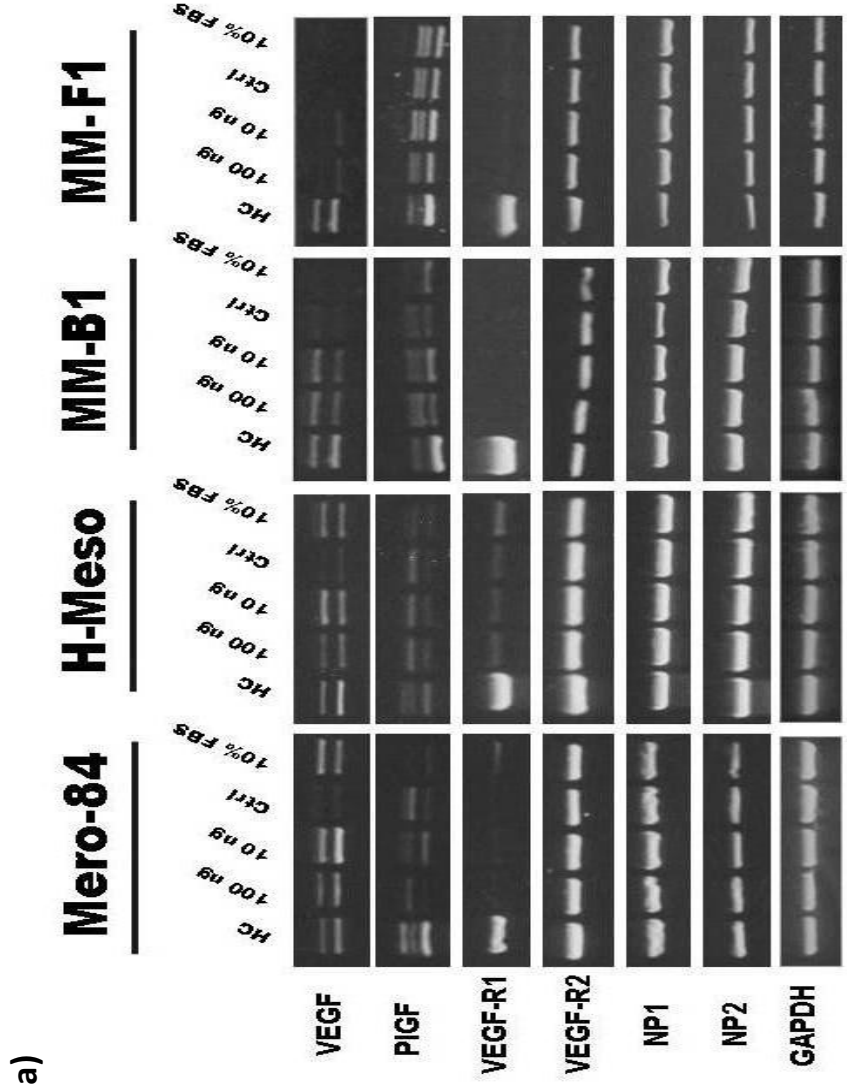

a)
IL-6
TGF-β
TNF-α
% Cytokines production
0
50
100
150
200
250
CTRL
PMA/IONO
MERO-84
H-MESO
MM-B1
MM-F1
b)
IL-6
TGF-β
TNF-α
% Cytokines production
0
50
100
150
200
250
300
CTRL
PMA/IONO
MERO-84
H-MESO
MM-B1
MM-F1

**Figure 5: Effects of MM-conditioned medium on Mo and Mϕ phenotype.**

Freshly isolated a) monocytes or b)macrophages were pre-incubated with MM-derived medium for 24 hours and then stimulated with 1 µM phorbol 12-myristate 13-acetate (PMA) and 10 µM ionomycin as reported in the Materials and Methods section. Data were acquired using a FACSCalibur running CellQuest software, and expressed as percentile production in respect to unstimulated (Ctrl) cells arbitrary set to 100. *p<0,05, as from ANOVA followed by Newman-Keuls test, in respect to PMA/IONO stimulated cells.

**Figure 6**


a)
Mero-84
H-Meso
MM-B1
MM-F1
HC
100 ng
10 ng
Ctrl
10% FBS
VEGF
PlGF
VEGF-R1
VEGF-R2
NP1
NP2
GAPDH

**b)**

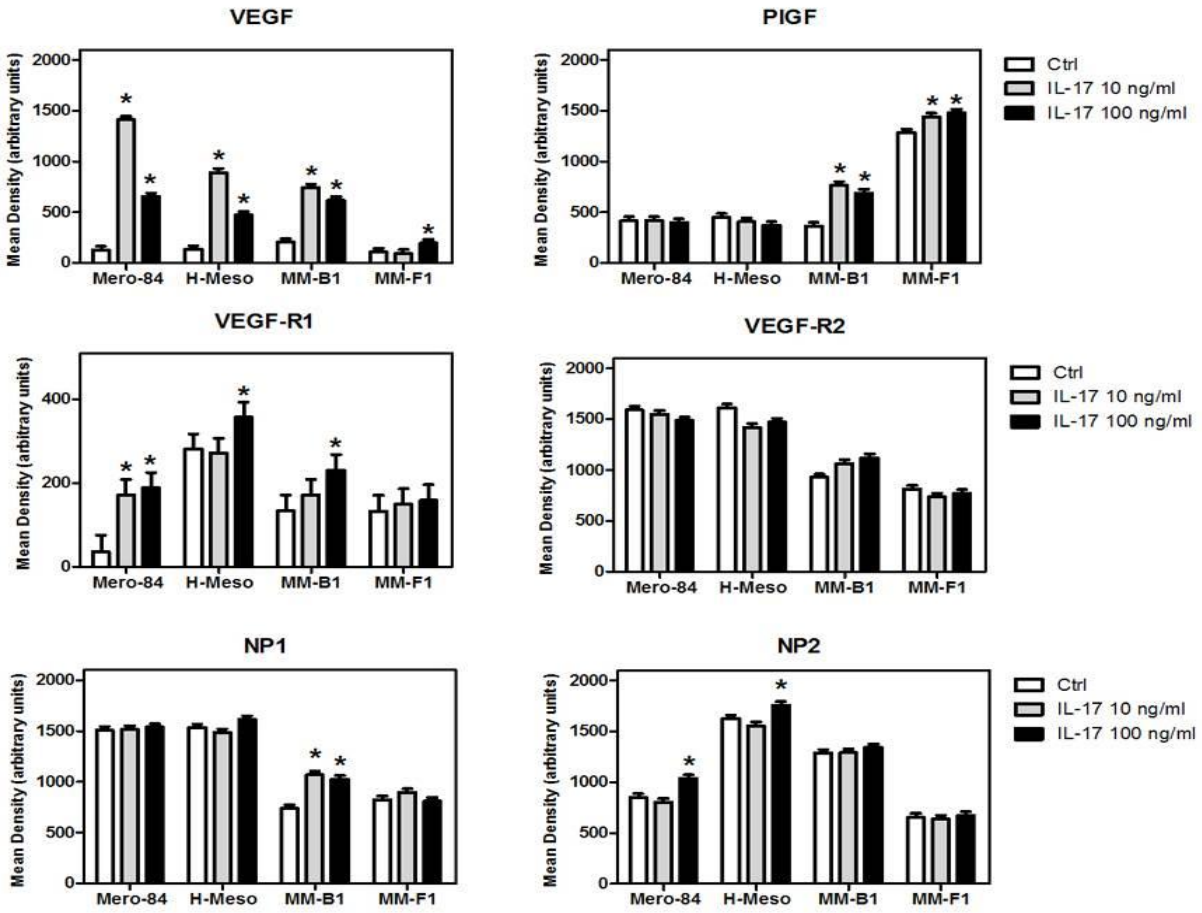
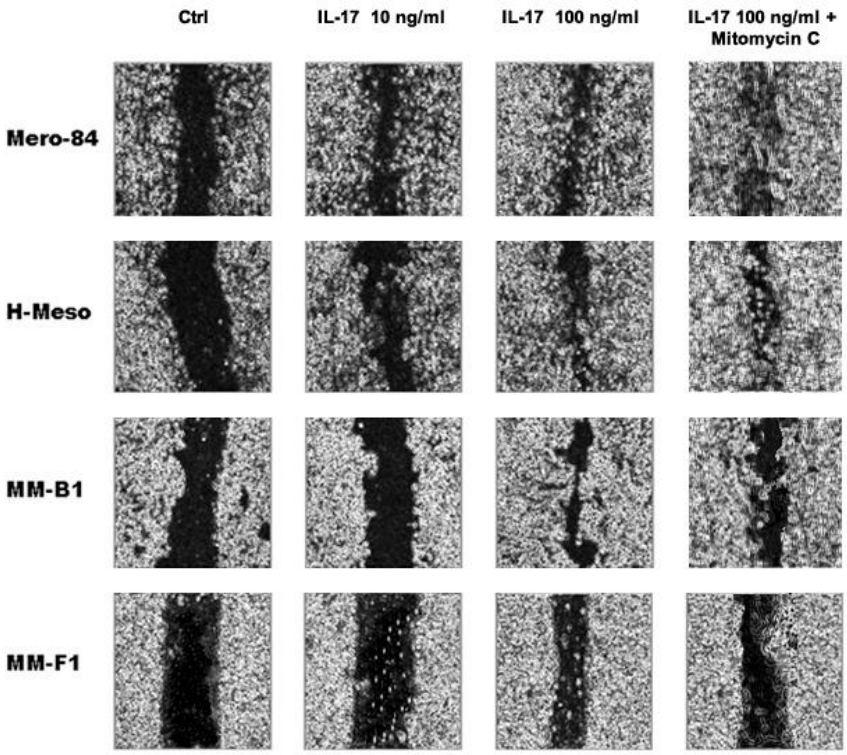

VEGF
PIGF
VEGF-R1
VEGF-R2
NP1
NP2
Mean Density (arbitrary units)
Mero-84
H-Meso
MM-B1
MM-F1
Ctrl
IL-17 10 ng/ml
IL-17 100 ng/ml

**Figure 6: Effects of IL-17 on the synthesis of VEGF and PlGF mRNAs.**

a) $1x10^5$ MM cells were seeded in RPMI medium supplemented with 0,2% BSA with or without different (10 or 100 ng/ml) IL-17 concentrations and incubated for 96 h. RNA extraction and PCR were then performed as reported in the Materials and Methods section. A significant experiment is shown, which was repeated two additional times with similar results. RNA from Huvec cells (HC) and MM cells incubated for 96 h in culture medium supplemented with 10% FBS were also run aside as internal controls.

b) Densitometrical analysis were performed using ImageJ software and data are presented as mean density ± S.D., reported as arbitrary units. *$p<0,05$, as from ANOVA followed by Newman-Keuls test, in respect to Ctrl (unstimulated) cells.

# Figure 7


Ctrl
IL-17 10 ng/ml
IL-17 100 ng/ml
IL-17 100 ng/ml + Mitomycin C
Mero-84
H-Meso
MM-B1
MM-F1

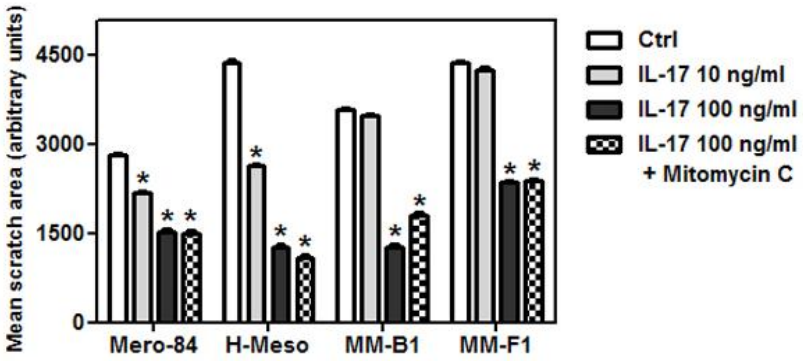

Mean scratch area (arbitrary units)
4500
3000
1500
0
Mero-84
H-Meso
MM-B1
MM-F1
Ctrl
IL-17 10 ng/ml
IL-17 100 ng/ml
IL-17 100 ng/ml + Mitomycin C

**Figure 7: Effects of IL-17 on the invasiveness of MM cells.**

2,5 x $10^5$ MM cells were plated in 6-well plates and scratches were performed as reported in the Materials and Methods section. The cells were then washed and incubated for 24 h in RPMI medium supplemented with 0,1% BSA with or without different (10 or 100 ng/ml) IL-17 concentrations plus or not Mitomycin C. MM cells were finally washed, stained with Giemsa staining and analyzed under inverted light microscope. Original magnification: 10 X. A significant experiment is shown, which was repeated two additional times with similar results. Mean scratch area, as desumed from three independent experiments and calculated by ImageJ software, is shown below. *p<0,05, as from ANOVA followed by Newman-Keuls test, in respect to Ctrl (unstimulated) cells.

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# Differential effects of malignant mesothelioma cells on THP-1 monocytes and macrophages

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**Abstract.** Malignant mesothelioma (MM) is a highly fatal tumor arising from inner body membranes, whose extensive growth is facilitated by its week immunogenicity and by its ability to blunt the immune response which should arise from the huge mass of leukocytes typically infiltrating this tumor. It has been reported that the inflammatory infiltrate found in MM tissues is characterized by a high prevalence of macrophages. Thus, in this work we evaluated the ability of human MM cells to modulate the inflammatory phenotype of human THP-1 monocytes and macrophages, a widely used *in vitro* model of monocyte/macrophage differentiation. Furthermore, we tested the hypothesis that the exposure to MM cells could alter the differentiation of THP-1 monocytes favoring the development of alternatively activated, tumor-supporting macrophages. Our data prove for the first time that MM cells can polarize monocytes towards an altered inflammatory phenotype and macrophages towards an immunosuppressive phenotype. Moreover, we demonstrate that monocytes cocultivated with MM cells 'keep a memory' of their encounter with the tumor which influences their differentiation to macrophages. On the whole, we provide evidence that MM cells exert distinct, cell-specific effects on monocytes and macrophages. The thorough characterization of such effects may be of a crucial importance for the rational design of new immunotherapeutic protocols.

## Introduction

Malignant mesothelioma (MM) is a rare tumor which originates from the mesothelial cell linings of the pleura and, less frequently, peritoneum and pericardium (1,2). Although it has a low metastatic efficiency, MM is highly invasive to surrounding tissues and its extensive growth leads to the failure of the organs underlying the serosal membranes (1,2). The median survival from diagnosis of MM is less than two years (2,3). Indeed, this aggressive tumor is seldom amenable to surgical intervention and poorly responsive to radiotherapy and chemotherapy (2,3). MM is also regarded as a weakly immunogenic tumor, whose ability to escape immune recognition relies on multiple mechanisms (4). Nonetheless, MM tissues are characterized by the presence of massive leukocyte infiltrates, mainly composed of macrophages (MΦ), natural killer cells and both $CD4^+$ and $CD8^+$ lymphocytes (4).

During recent years, substantial evidence has indicated that under the pressure of tumor microenvironmental factors including growth factors, cytokines and prostaglandins, tumor-infiltrating leukocytes can undergo a dramatic shift in their activities, aborting immunosurveillance and starting to actively support tumor growth, angiogenesis and tissue remodeling (5,6). Tumor-associated MΦ (TAMs) appear to play a major role in this regard (7,8). It is well established that MΦ can act as both positive or negative regulators of the immune system so that, depending on their pro- or anti-inflammatory functional program, mature MΦ are classified as M1, or 'classically activated', or as M2, or 'alternatively activated'. M1 MΦ are powerful immune effector cells characterized by a high ability to present antigens and to produce pro-inflammatory cytokines and toxic intermediates, while M2 MΦ display a poor antigen-presenting capacity and are polarized to release many different immunosuppressive molecules as well as to promote angiogenesis and extracellular matrix degradation (6-9). It has been indicated that TAMs, which derive almost entirely from circulating monocytes (Mo) recruited and differentiated in the tumor milieu (10-12), often display phenotypic features consistent with those of M2 MΦ and, accordingly, can actively sustain tumor growth and contribute to generate an immunosuppressive microenvironment (7,13). Moreover, recent evidence demonstrated the existence of Mo with M2-like phenotypes, which could thus be a preferential source of M2 MΦ and important players in tumor-induced immunosuppression (10,12).

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The definition of the role of individual immune cell types on the outcome of the anti-tumor immune responses is of pivotal importance for the rational design of new antineoplastic immunotherapy approaches. In this respect, however, the functional role of the leukocytes infiltrating MM tissues is still poorly defined (4). Thus, prompted by the reported abundance of infiltrating MΦ in MM, we evaluated the ability of human MM cells to modulate the inflammatory phenotype of the THP-1 cell lineage, a widely used *in vitro* model of Mo/MΦ differentiation (14). Furthermore, we tested the hypothesis that the exposure to MM cells could alter the differentiation of THP-1 Mo favoring the development of alternatively activated, tumor-supporting MΦ.

## Materials and methods

*Cell cultures*. The human monocytic cell line THP-1 (15) was obtained from the American Type Culture Collection (Manassas, VA, USA). The human pleural MM cell line Mero 84 (16) was a generous gift of Dr Marjan Versnel. Both cell lines were cultured at 37˚C in 5% $CO_2$ in DMEM supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 $\mu$g/ml streptomycin, 100 U/ml penicillin and 100 $\mu$g/ml sodium pyruvate (complete medium), all from Cambrex (Lonza Milano, Milan, Italy). THP-1 Mo were differentiated to MΦ by incubation with 100 nM phorbol 12-myristate 13-acetate (PMA; Sigma Aldrich, Milan, Italy) for 72 h in the dark, as previously described (17).

*Experimental plan*. The effects of MM cells on the phenotype of THP-1 Mo were evaluated using the following experimental plan: THP-1 Mo were seeded in complete medium in 6-well plates at a density of $1\text{-}3x10^5$ cells/well; each well was then covered with a cell culture insert (pore size: 0.4 $\mu$M; Sigma Aldrich) containing an equal amount of Mero 84 cells seeded in the same medium (THP-1/Mero 84 cell ratio 1:1); after 24, 48 or 72 h of cocultivation, the inserts were removed and the Mo were washed, counted and replated. The cultures containing Mero 84-exposed Mo (Mo-MM) and control cultures containing an equal number of Mo not exposed to Mero 84 cells were then stimulated with 5 $\mu$g/ml of LPS (Sigma-Aldrich) for 24 h and used for comparative phenotypic evaluations.

The effects of MM cells on the phenotype of THP-1 MΦ were evaluated using two different experimental settings: in the first setting, THP-1 Mo were differentiated to MΦ and then cocultivated with Mero 84 cells; in the second setting, Mo were first cocultivated with Mero 84 cells and then differentiated to MΦ. Cocultures were performed as described above, with a THP-1/Mero 84 cell ratio of 1:1. Equal numbers of MΦ, Mero 84-exposed MΦ (MΦ1-MM) and MΦ generated from Mero 84-exposed Mo (MΦ2-MM) were then plated and stimulated with 5 $\mu$g/ml of LPS for 24 h. The cultures were finally used for comparative phenotypic evaluations as described below.

The growth and survival rate of THP-1 Mo and MΦ cultures were routinely assessed by cell counts using the trypan blue exclusion method; proliferation and survival of THP-1 Mo and MΦ were found to be unaffected by the cocultivation with MM cells and by LPS treatment (data not shown).

*[$^3$H]-arachidonic acid release assay*. THP-1 Mo were labelled for 3 h with 1 $\mu$Ci [$^3$H]-arachidonic acid ([$^3$H]-AA, specific activity 202,4 Ci/mmol; Perkin-Elmer Italia, Monza, Italy) at 37˚C in serum-free medium, as previously described (18). The labelled cells were then washed twice in serum-free medium to remove unincorporated [$^3$H]-AA and used to obtain replicate cultures of labelled Mo, MΦ, Mo-MM, MΦ1-MM and MΦ2-MM. The cultures were finally stimulated with LPS for 24 h. Cultures containing [$^3$H]-AA-labelled, LPS-unstimulated Mo and MΦ were used to control basal [$^3$H]-AA release. Culture media were collected and 100 $\mu$l aliquots were added to 3 ml Optifluor (Perkin-Elmer Italia) and analyzed by a liquid scintillator counter.

*Measurement of prostaglandin $E_2$, tumor necrosis factor-α and interleukin-10 release*. The amounts of prostaglandin $E_2$ ($PGE_2$), tumor necrosis factor-α (TNF-α) and interleukin-10 (IL-10) released in the culture media by LPS-stimulated Mo, MΦ, Mo-MM, MΦ1-MM and MΦ2-MM and by LPS-unstimulated Mo and MΦ, were quantified using a monoclonal $PGE_2$ EIA kit (Cayman Chemicals, Italy Cabru, Arcore, Italy), an ELISA kit for TNF-α and an ELISA kit for IL-10 (both from Pierce Endogen, Celbio, Milan, Italy) according to the manufacturer's instructions.

*Evaluation of nitrite production by Griess reaction*. The amount of nitrite ($NO_2$) released in the culture media of LPS-stimulated Mo, MΦ, Mo-MM, MΦ1-MM and MΦ2-MM and of LPS-unstimulated Mo and MΦ was quantified as previously described (19). Briefly, equal volumes of culture media and Griess modified reagent (Sigma-Aldrich) were mixed in a macrotitre plate. Upon incubation for 30 min at room temperature, the presence of $NO_2$ in the samples determines the formation of a fluorescent product sensible to stimulation with a laser beam set at 550 nm. $NO_2$ concentration in each sample was thus determined by plotting the sample OD at 550 nm on a standard curve prepared with sodium nitrite solutions (concentration range 0.43-65 $\mu$M).

*Phagocytosis assay*. The phagocytic activity of THP-1 cells was evaluated by measuring the uptake of FITC-dextran particles by flow cytometry, as previously described (20). In brief, THP-1 Mo, MΦ, Mo-MM, MΦ1-MM and MΦ2-MM were stimulated with LPS in the presence of 1 mg/ml FITC-Dextran (MW: 70 kDa; Sigma-Aldrich) for 24 h in the dark. Control Mo and MΦ were also incubated with FITC-Dextran in the absence of LPS, in order to assess basal phagocytic activity. At the end of incubation, the cells were extensively washed, recovered by centrifugation and analyzed by FACS.

*Collagenolytic activity assay*. The serum-free culture media of LPS-stimulated Mo, MΦ, Mo-MM, MΦ1-MM and MΦ2-MM and of LPS-unstimulated Mo and MΦ were collected and 10-fold concentrated using Centricon devices (cut-off: 10 kDa; Millipore, Milan, Italy). Aliquots (200 $\mu$l) of concentrated

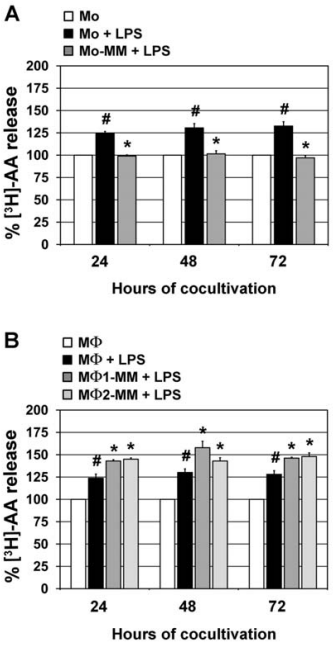


Figure 1. Effects of MM cells on the release of [$^3$H]-AA by THP-1 Mo and MΦ. (A) Percentile mean ± SD of [$^3$H]-AA release by unstimulated Mo, LPS-stimulated Mo and LPS-stimulated Mo-MM obtained by coculturing Mo with MM cells for 24, 48 and 72 h. (B) Percentile mean ± SD of [$^3$H]-AA release by unstimulated MΦ, LPS-stimulated MΦ, LPS-stimulated MΦ1-MM obtained by coculturing MΦ with MM cells for 24-72 h, and LPS-stimulated MΦ2-MM generated by Mo cocultured with MM cells for 24-72 h and then differentiated to MΦ. $^{\#}p<0.05$ vs. unstimulated Mo or MΦ and $^{*}p<0.05$ vs. LPS-stimulated Mo or MΦ, as evaluated by ANOVA followed by Newman-Keuls test.

media were mixed with 100 $\mu$l of fluorescein-labelled collagen (final concentration: 2.5 mg/ml; Sigma-Aldrich) and incubated for 2 h in the dark at 37˚C. Each mixture was brought to a final volume of 2 ml with phosphate-buffered saline (PBS) and the sample fluorescence was analyzed using a computer-aided background-controlling fluorimeter (Perkin-Elmer LS 50 b), with the excitation wavelength set at 485 nm, the emission wavelength set at 530 nm, and slits, respectively, set at 5 and 10 nm for each light pathway (21).

*Statistical analysis*. Data distribution was preliminarily verified by the Kolmogorov-Smirnov test, and data sets were analyzed by One-way analysis of variance (ANOVA) followed by Newman-Keuls test. Differences were regarded as significant when p-value was <0.05. Quantitative data were expressed as the percentile mean ± SD of at least four replicate determinations, each performed in triplicate, in respect to control levels arbitrary set to 100, except where otherwise indicated.

## Results

*Effects of MM cells on the release of [$^3$H]-AA by THP-1 Mo and MΦ*. The release of AA from membrane phospholipids is an important marker of Mo and MΦ activation (22-24). Therefore, in order to characterize the possible immuno-modulatory effects exerted by MM cells on the THP-1 lineage, we first evaluated whether the cocultivation with Mero 84 cells could affect the release of [$^3$H]-AA by LPS-stimulated THP-1 Mo and MΦ.

As illustrated in Fig. 1A, LPS-stimulated Mo-MM released less [$^3$H]-AA than LPS-stimulated, non-cocultivated Mo. In particular, the amounts of [$^3$H]-AA released by LPS-stimulated Mo-MM generated by coculturing THP-1 Mo with Mero 84 cells for 24, 48 or 72 h were similar to those released by unstimulated Mo, and about 25% lower than those of LPS-stimulated Mo. Conversely, as illustrated in Fig. 1B, MΦ1-MM obtained by coculturing MΦ with MM cells for 24-72 h released significantly more [$^3$H]-AA than non-cocultivated MΦ in response to LPS. Moreover, also the LPS-stimulated MΦ2-MM generated from Mo exposed to MM cells for 24-72 h released higher amounts of [$^3$H]-AA as compared to LPS-stimulated, non-cocultivated MΦ. Since the effects observed after 24-72 h of cocultivation were similar, a 24 h cocultivation time was chosen for all subsequent experiments.

*Effects of MM cells on $PGE_2$ release by THP-1 Mo and MΦ*. $PGE_2$ is one of the major AA metabolites and it is known to play immunosuppressive functions in the tumor micro-environment (6,25). Thus, it was evaluated whether the cocultivation with Mero 84 cells could modulate the release of $PGE_2$ by LPS-stimulated THP-1 Mo and MΦ.

Consistent with the results obtained in [$^3$H]-AA release studies, the cocultivation with MM cells had opposite effects on the release of $PGE_2$ by THP-1 Mo and MΦ. Indeed, as shown in Fig. 2, LPS-stimulated Mo-MM released lower amounts of $PGE_2$ as compared to non-cocultivated, LPS-stimulated Mo, while the amounts of $PGE_2$ released upon LPS stimulation by both MΦ1-MM and MΦ2-MM were higher than those released by non-cocultivated MΦ.

*Differential effects of MM cells on TNF-α and IL-10 release*. To further evaluate the ability of MM cells to modulate the phenotype of THP-1 cells, experiments were conducted to assess whether the coculture with Mero 84 cells could affect the release of the prototypical pro-inflammatory cytokine TNF-α and the prototypical anti-inflammatory cytokine IL-10 (13) by THP-1 Mo and MΦ. Data in Fig. 3A show that the release of TNF-α induced by LPS was about ten-fold lower in Mo-MM vs. the non-cocultivated Mo, while the amounts of IL-10 released in response to LPS by Mo and Mo-MM were similar. Conversely, data in Fig. 3B show that non-cocultivated MΦ, MΦ1-MM and MΦ2-MM released comparable amounts of TNF-α upon LPS stimulation, whereas both MΦ1-MM and MΦ2-MM showed higher levels of LPS-induced IL-10 release than non-cocultivated MΦ.

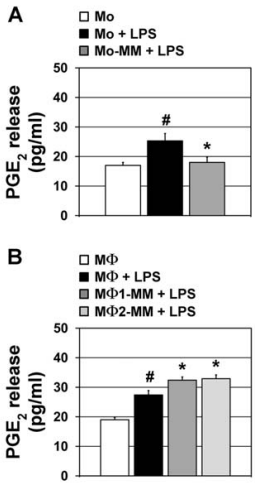


Figure 2. Effects of MM cells on $PGE_2$ release by THP-1 Mo and MΦ. (A) Mean ± SD values of $PGE_2$ release (pg/ml) by unstimulated Mo, LPS-stimulated Mo and LPS-stimulated Mo-MM obtained by coculturing Mo with MM cells for 24 h. (B) Mean ± SD values of $PGE_2$ release (pg/ml) by unstimulated MΦ, LPS-stimulated MΦ, LPS-stimulated MΦ1-MM obtained by coculturing MΦ with MM cells for 24 h, and LPS-stimulated MΦ2-MM generated by Mo cocultured with MM cells for 24 h and then differentiated to MΦ. $^{\#}p<0.05$ vs. unstimulated Mo or MΦ and $^{*}p<0.05$ vs. LPS-stimulated Mo or MΦ, as evaluated by ANOVA followed by Newman-Keuls test.

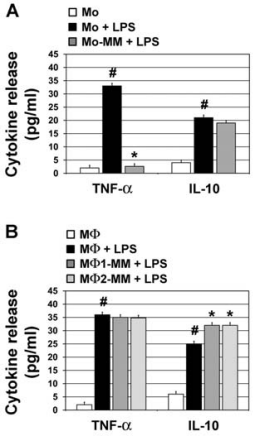


Figure 3. Effects of MM cells on TNF-α and IL-10 release. (A) Mean ± SD values of TNF-α and IL-10 release (pg/ml) by unstimulated Mo, LPS-stimulated Mo and LPS-stimulated Mo-MM obtained by coculturing Mo with MM cells for 24 h. (B) Mean ± SD values of TNF-α and IL-10 release (pg/ml) by unstimulated MΦ, LPS-stimulated MΦ, LPS-stimulated MΦ1-MM obtained by coculturing MΦ with MM cells for 24 h, and LPS-stimulated MΦ2-MM generated by Mo cocultured with MM cells for 24 h and then differentiated to MΦ. $^{\#}p<0.05$ vs. unstimulated Mo or MΦ and $^{*}p<0.05$ vs. LPS-stimulated Mo or MΦ, as evaluated by ANOVA followed by Newman-Keuls test.

*Effects of MM cells on $NO_2$ production*. According to different authors, the polarization towards an M2 phenotype is accompanied by a reduction in the capacity to produce NO (7,26,27). In order to assess whether the cocultivation with MM cells could affect the production of NO by THP-1 Mo and MΦ, the concentration of $NO_2$ in culture media was measured as a surrogate of NO production (19,28). Indeed $NO_2$ is the major metabolite of NO and its extracellular concentration is directly proportional to that of NO (19).

Data in Fig. 4 show that upon LPS stimulation Mo and Mo-MM produced similar amounts of $NO_2$. On the other hand, both MΦ1-MM and MΦ2-MM produced lower levels of $NO_2$ than non-cocultivated MΦ.

*Effects of MM cells on Mo and MΦ phagocytic activity*. Next, we focused on the effects of MM cells on the phagocytic properties of THP-1 Mo and MΦ. Data in Fig. 5A show that LPS-stimulated Mo-MM displayed a small increase in the ability to phagocytize FITC-dextran particles as compared to non-cocultivated, LPS-stimulated Mo. However, as illustrated in Fig. 5C this increase did not reach statistical significance. On the other hand, as illustrated in Fig. 5B-D, the phagocytic activity of both MΦ1-MM and MΦ2-MM was significantly lower than that of non-cocultivated MΦ upon LPS-stimulation,

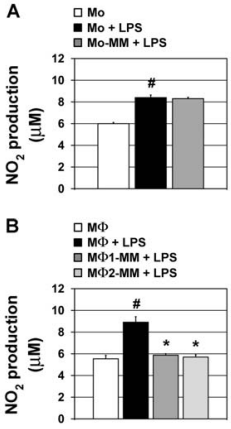


Figure 4. Effects of MM cells on $NO_2$ production. (A) Mean ± SD values of $NO_2$ ($\mu$M) produced by unstimulated Mo, LPS-stimulated Mo and LPS-stimulated Mo-MM obtained by coculturing Mo with MM cells for 24 h. (B) Mean ± SD values of $NO_2$ ($\mu$M) produced by unstimulated MΦ, LPS-stimulated MΦ, LPS-stimulated MΦ1-MM obtained by coculturing MΦ with MM cells for 24 h, and LPS-stimulated MΦ2-MM generated by Mo cocultured with MM cells for 24 h and then differentiated to MΦ. $^{\#}p<0.05$ vs. unstimulated Mo or MΦ and $^{*}p<0.05$ vs. LPS-stimulated Mo or MΦ, as evaluated by ANOVA followed by Newman-Keuls test.

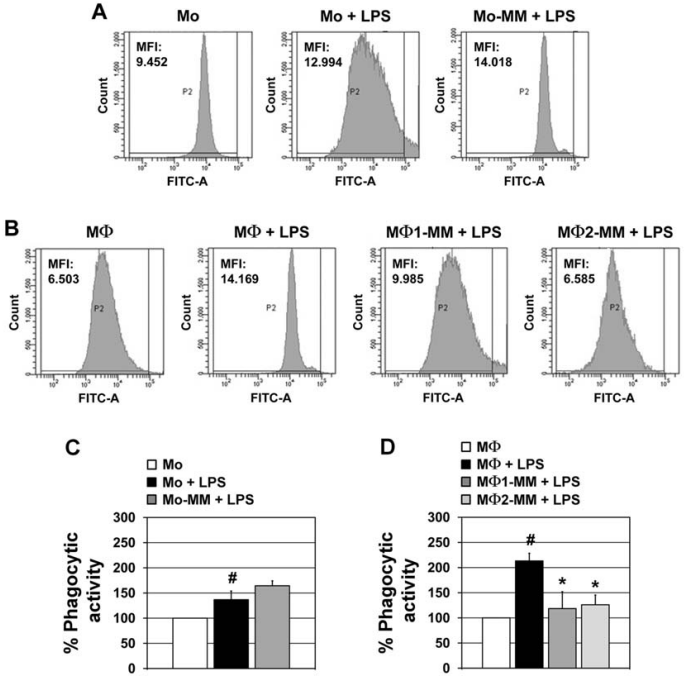


Figure 5. Effects of MM cells on Mo and MΦ phagocytic activity as assessed by flow cytometric analysis of FITC-dextran uptake. (A) A representative determination of FITC-dextran uptake by unstimulated Mo, LPS-stimulated Mo and LPS-stimulated Mo-MM obtained by coculturing Mo with MM cells for 24 h; the mean fluorescence intensity (MFI) is indicated in each case. (B) A representative determination of FITC-dextran uptake by unstimulated MΦ, LPS-stimulated MΦ, LPS-stimulated MΦ1-MM obtained by coculturing MΦ with MM cells for 24 h, and LPS-stimulated MΦ2-MM generated by Mo cocultured with MM cells for 24 h and then differentiated to MΦ; the MFI is indicated in each case. (C) Percentile mean ± SD of the phagocytic activity of unstimulated Mo, LPS-stimulated Mo and LPS-stimulated Mo-MM as determined from four different FITC-dextran uptake experiments. (D) Percentile mean ± SD of the phagocytic activity of unstimulated MΦ, LPS-stimulated MΦ, LPS-stimulated MΦ1-MM and MΦ2-MM as determined from four different FITC-dextran uptake experiments. #p<0.05 vs. unstimulated Mo or MΦ and *p<0.05 vs. LPS-stimulated Mo or MΦ, as evaluated by ANOVA followed by Newman-Keuls test.

Table I. Differential effects of MM cells on the phenotype of LPS-stimulated THP-1 Mo and MΦ.

| | Mo-MM vs. Mo | MΦ1-MM vs. MΦ | MΦ2-MM vs. MΦ |
|---|---|---|---|
| AA release | - | + | + |
| $PGE_2$ release | - | + | + |
| TNF-α release | - | ns | ns |
| IL-10 release | ns | + | + |
| $NO_2$ production | ns | - | - |
| Phagocytic activity | ns | - | - |
| Collagenolytic activity | + | + | + |

+, significantly increased; -, significantly decreased; ns, not significantly modified.

being almost equal to that of unstimulated, non-cocultivated MΦ.

*MM cells up-regulate the collagenolytic activity of both THP-1 Mo and MΦ.* Finally, it was assessed whether the cocultivation with MM cells could modulate collagen degradation by THP-1 Mo and MΦ. Interestingly, MM cells exerted comparable effects on the collagenolytic activity of the two cell types. Indeed, as illustrated in Fig. 6, the collagenolytic activity of LPS-stimulated Mo-MM was nearly doubled as compared to that of the non-cocultivated, LPS-stimulated Mo and, similarly, the collagenolytic activity of LPS-stimulated MΦ1-MM and MΦ2-MM was more than doubled as compared to that of the non-cocultivated, LPS-stimulated MΦ. The reported effects exerted by MM cells on the phenotype of THP-1 Mo and MΦ are summarized in Table I.

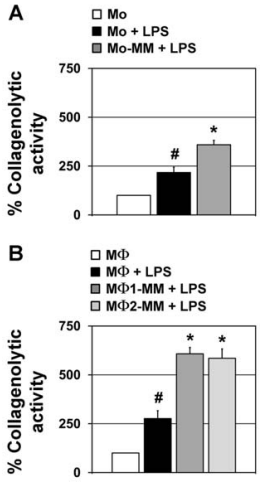


Figure 6. MM cells up-regulate the collagenolytic activity of THP-1 Mo and MΦ. (A) Percentile mean ± SD of the collagenolytic activity of unstimulated Mo, LPS-stimulated Mo and LPS-stimulated Mo-MM obtained by coculturing Mo with MM cells for 24 h. (B) Percentile mean ± SD of the collagenolytic activity of unstimulated MΦ, LPS-stimulated MΦ, LPS-stimulated MΦ1-MM obtained by coculturing MΦ with MM cells for 24 h, and LPS-stimulated MΦ2-MM generated by Mo cocultured with MM cells for 24 h and then differentiated to MΦ. $^{\#}p<0.05$ vs. unstimulated Mo or MΦ and $^{*}p<0.05$ vs. LPS-stimulated Mo or MΦ, as evaluated by ANOVA followed by Newman-Keuls test.

## Discussion

This report represents the first direct evidence of the broad immunomodulatory effects exerted by pleural MM cells on Mo and MΦ. Mononuclear phagocytes are among the earliest immune cells infiltrating tumor sites and they nominally take part to almost all the phases of the anti-tumor reaction (29). In fact, tumor-infiltrating Mo can effectively switch on the inflammatory reaction by releasing chemoattractant and immunostimulating factors and can differentiate into MΦ, thus empowering the innate response while bridging to the acquired response (29). On the other hand, tumors can develop several strategies to counteract immune effector cells and escape recognition and destruction by the immune system. For instance, many of the soluble factors occurring in the cancer microenvironment are able to suppress the anti-tumor functions of Mo and MΦ or even to alter Mo/MΦ functional programs, thus transforming these cells into powerful tumor's allies (5,29).

In the attempt to shed light upon the immunomodulatory activities of MM, we developed a cocultivation framework to evaluate the effects of MM cells on the phenotype of both Mo and MΦ of the THP-1 lineage. Furthermore, we evaluated whether MM cells could polarize Mo development into immunosuppressive MΦ.

The first evidence we provide for the immunomodulatory properties of MM cells is represented by their effects on the release of [$^3$H]-AA by THP-1 Mo and MΦ. Indeed our data demonstrate that the cocultivation with MM cells blunts the release of [$^3$H]-AA by Mo in response to an inflammatory stimulus, while it conversely polarizes MΦ to over-release [$^3$H]-AA in response to the same stimulus. Following its release, most AA is metabolized to a large family of pro- and anti-inflammatory metabolites in a cell-specific manner (25,30). When the LPS-induced release of $PGE_2$, a major AA metabolite with anti-inflammatory properties (25,31) was assessed, it resulted that after the cocultivation with MM cells the amount of $PGE_2$ released by Mo was significantly reduced while, at the opposite, MΦ displayed a significant up-regulation of $PGE_2$ release. Interestingly, it has been reported that MM tissues show high levels of $PGE_2$ (32), and that $PGE_2$ production in tumor sites can promote the development of regulatory T cells (Tregs), which in turn dramatically hamper the effectiveness of the anti-tumor immune response (33).

Further evidence of the differential immunomodulatory effect exerted by MM on the Mo/MΦ lineage is represented by the cytokine profile expressed by THP-1 Mo and MΦ after exposure to MM cells. Following cocultivation with MM cells, the LPS-induced release of the prototypic M1 cytokine TNF-α (13) was markedly decreased in Mo cultures, while it appeared to be unaffected in MΦ cultures. Conversely, the cocultivation with MM cells enhanced the release of the prototypic M2 cytokine IL-10 (13) by LPS-stimulated MΦ, but did not modify the amount of IL-10 released by LPS-stimulated Mo. Moreover, as assessed through the measurement of $NO_2$, the LPS-stimulated release of NO, which is one of the most important tumoricidal molecules produced by the Mo/MΦ lineage (26), was not affected in Mo cocultivated with MM cells, whereas it was significantly reduced in MM-exposed MΦ. Collectively, these data indicate that MM cells alter the phenotypic properties of Mo, while more clearly shifting MΦ towards an M2-like, protumoral phenotype (10,13,34). The increased production of the immunosuppressive $PGE_2$ and IL-10 and the decrease of NO release observed in MM-exposed MΦ are both consistent with a shift towards the acquisition of M2 features (7,13,27,31). Still, this MΦ polarization towards an immunosuppressive phenotype was not associated with a reduction in the release of TNF-α. Even though TNF-α is considered as a prototypical M1 cytokine, it also displays several tumor-supporting properties, including the ability to promote tumor growth, migration, invasion and angiogenesis (35). Moreover, the production of such a molecule in developing MM foci is thought to mainly depend on MΦ and it has been linked to the malignant transformation of mesothelial cells (36). Thus, the observation that the LPS-induced release of TNF-α was not reduced in MM-exposed MΦ indicates that, while acquiring immunosuppressive properties, these cells maintain the expression of pro-inflammatory, tumor-supporting mediators.

We also observed that the cocultivation with MM cells had different effects on the phagocytosis of dextran particles by LPS-stimulated Mo and MΦ: the phagocytic activity of

MM-exposed Mo was similar to that of non-cocultivated Mo, whereas the phagocytic activity of MM-exposed MΦ was significantly reduced as compared to that of non-cocultivated MΦ. In view of the results discussed so far, this finding is peculiar. Indeed, according to different authors, alternatively activated MΦ exhibit a poor antigen-presenting ability but a high capacity for phagocytosis and, accordingly, are efficient scavengers of apoptotic cells and cell debris (7,37). On the other hand, an inefficient clearance of apoptotic cells can elicit pro-inflammatory as well as pro-tumoral, immunosuppressive responses (6). Therefore, the inhibition of the phagocytic activity observed in MM-exposed MΦ may play a double-edged role in the modulation of immune functions within the tumor microenvironment.

The observed ability of MM cells to decrease the release of TNF-α and $PGE_2$ without altering the production of IL-10 and $NO_2$ by THP-1 Mo indicates the instauration of an altered phenotype in Mo, which exhibit mixed pro- and anti-inflammatory features. It has been reported that tumor-associated Mo can exhibit immunosuppressive, M2-like properties. On the other hand, according to different authors, such Mo appear to display complex and multifaceted phenotypes and may develop from Mo undergoing a transient phase of activation followed by a phase of refractoriness to inflammatory stimuli (10,34,38,39). The altered phenotype displayed by MM-exposed Mo may thus represent an intermediate stage toward the acquisition of more defined immunosuppressive properties.

Remarkably, as assessed by the release of lipid mediators ([$^3$H]-AA, $PGE_2$), cytokines (TNF-α, IL-10) and NO as well as by the phagocytic and collagenolytic activity, the phenotype of THP-1 MΦ cocultivated with MM cells (MΦ1-MM) was almost identical to that of MΦ generated by Mo cocultivated with MM cells (MΦ2-MM) for 24 h. This finding demonstrates for the first time that MM-exposed Mo are a 'developmentally polarized' cell type, being yet committed to an altered differentiation after 24 h of cocultivation with MM cells. These Mo differentiated into immunosuppressive MΦ in the absence of any further MM-derived factor added during the differentiation process, supporting the hypothesis that altered Mo are a privileged source of M2 MΦ.

Collagen degradation is crucially involved in the formation of new vessels as well as in tumor cell migration and invasion (40). Unlike the differential effects discussed above, the cocultivation with MM cells induced a strong up-regulation of the collagen-degrading activity in both LPS-stimulated Mo and MΦ, demonstrating that either of these cell types can be co-opted by the tumor as an effector of extracellular matrix degradation and tissue remodeling.

Taken together, our data demonstrate that MM polarizes Mo and MΦ towards distinct phenotypes and that Mo 'keep a memory' of their encounter with the tumor which influences their development to MΦ. By suggesting to target Mo in addition to MΦ (41) for the reversal of tumor-supporting immune cell phenotypes, our data may bear significance for the design of future immunotherapy approaches for MM.

The mechanisms by which tumor cells educate immune cells to exert tumor-supporting functions have not been fully elucidated to date (10). The THP-1/MM cells coculture model could represent a valuable system to investigate the hierarchic role of MM-released factors involved in the modulation of the phenotype of Mo and MΦ.

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# ENDOMORPHIN-1 INHIBITS THE ACTIVATION AND THE DEVELOPMENT OF A HYPORESPONSIVE-LIKE PHENOTYPE IN LIPOPOLYSACCHARIDE- STIMULATED THP-1 MONOCYTES

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**Endomorphin-1 (EM-1) is an endogenous opioid peptide selectively binding to μ opioid receptors (MORs). Besides its analgesic effects on the central nervous system (CNS), it has been recently reported that EM-1 can cross the blood-brain barrier (BBB) and diffuse into the blood, behaving as an analgesic/anti-inflammatory molecule on peripheral tissues, thus leading to the hypothesis that it could represent a soluble modulator of immune cell functions. Interestingly, nothing is known about its possible effects on monocytes, the main circulating cell-type involved in those systemic responses, such as fever and septic states, involving the release of high amounts of pyrogenic inflammatory factors. The aim of this work is to evaluate possible EM-1effects on lipopolisaccharide (LPS)-stimulated THP-1 monocytes in terms of the production of inflammatory mediators and the instauration of a hyporesponsive-like phenotype which is a main feature of systemic inflammatory responses, and on the development of peripheral monocytes to DC. Our data demonstrate for the first time that EM-1 is able to inhibit both LPS-stimulated monocyte activation, in terms of arachidonic acid, $PGE_2$, ROI and $NO_2$ production and instauration of a hyporesponsive phenotype without any macroscopic effect on DC development. These data support the hypothesis that EM-1 could be involved in modulating monocyte functions during systemic inflammatory reactions, also providing new evidence for its eventual clinical application in endotoxic states.**

Endomorphin-1 (EM-1) is an ammidated tetrapeptide discovered by Zadina et al. in human tissues mostly belonging to the nervous and the immune system (1-2). To date, EM-1 is thought to be a powerful endogenous analgesic molecule, as its presence in the nervous system has been traced in those areas involved in pain perception (nociception) and in the spinal antinociceptive descending pathways (2-3). Notably, it has also been recently demonstrated that EM-1 can cross the blood-brain barrier (BBB), thus entering the blood flow and possibly reaching areas of the body far

*Key words: monocytes, endomorphin, opioid, inflammation, hyporesponsivity*

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from the central nervous system (4-5). Coherently, EM-1 presence has been traced in many peripheral inflamed tissues, where it has proven to significantly decrease sensory neuron firing rate (6-7) and, most notably, to significantly suppress the release of inflammatory factors from macrophages, which mainly accounts for the development of any algesic reaction occurring in peripheral tissues (6).

Even though this evidence allows to hypothesize a new role for EM-1 as a neuro-endocrine regulator of immune functions, it still lacks any knowledge about its possible effects on other leukocytes expressing μ opioid receptors (MORs) (7). Among these, monocytes would be a favorable target for EM-1 anti-inflammatory activity, for they are the major cell type involved in systemic inflammatory reactions having a key role in inducing fever and Systemic Inflammatory Reaction Syndrome (SIRS) (8-9). In fact, when monocytes are stimulated by high amounts of pathogen products (as happens in septic states), an excessive intravascular release of inflammatory mediators such as prostaglandins (PGs), leukotrienes (LTs) and oxidant molecules can result in multiple organ failure and host death (8-10).

Moreover, an additional alteration of monocyte functions can occur during septic states: as a consequence of a profound inflammatory activation, in fact, monocytes can develop a hyporesponsive phenotype, which makes them insensitive to new stimulation (11). Such a phenomenon, resulting in a diffused immunoparalytic state, is also detrimental as it exposes the host to a facilitated colonization by invading pathogens (11). Finally, as a further alteration of monocyte functions during septic states, it has been recently postulated that the excessive activation of these cells could impair their development to dendritic cells (DCs), the major antigen-presenting cells (APCs), which would then spread immunoparalysis to the specific compartment of the immune response (10, 12-13).

Thus, in the view of a supposed role for EM-1 as a new immunosuppressive molecule, the aim of this work is to investigate possible EM-1 effects on a model of intense monocyte activation consisting of human THP-1 cells pulsed with high lipopolysaccharide (LPS) doses in order to reproduce *in vitro* a situation comparable to an *in vivo* septic state. In this context, this study evaluates EM-1 effects on the production of systemic inflammatory mediators such as PGs, LTs and Reactive Oxygen and Nitrogen Intermediates (ROI and NOI, respectively), also assessing its possible effects in preventing the development of monocyte hyporesponsivity and in driving monocyte differentiation to DCs.

## MATERIALS AND METHODS

*Cell culture*

The human monocytic cell line THP-1 was obtained from American Type Culture Collection (Manassas, VA) and cultured at 37°C in 5% CO2 with RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), L-glutamine (2 mM), streptomycin (100 μg/ml), penicillin (100 U/ml), sodium pyruvate (100 μg/ml), herein referred to as "complete medium". In all experiments, to determine cell viability, monocyte cultures were stained with trypan blue at specific time-points after treatment with different reagents. Stained vs living cells were counted under a microscope using a Neubauer modified chamber.

*[$^3$H]-Arachidonic acid release assays*

1x10$^6$ THP-1 cells/well were seeded in complete medium and labelled for 3 h with 1 μCi [$^3$H]-arachidonic acid (AA, spec. act. 202,4 Ci/mmol) at 37°C, as previously described (14). To remove non-specific binding of [$^3$H]-AA to cell surface prior to agonist stimulation, cells were recollected by centrifugation at 1200 rpm for 5 min and washed twice in serum-free medium prior to re-suspension in fresh complete medium. In experiments involving a single LPS pulse, cells were stimulated with different LPS (1-10 μg/ml) and EM-1 concentrations ($10^{-6}$ – $10^{-12}$M) for 24 h. When requested, cells were also incubated with 10 μM methyl arachydonil fluorophosphate (MAFP, a selective cPLA$_2$ inhibitor) for 30 min, with $10^{-8}$M diphenylene iodinium (DPI, a specific NADPH oxidase inhibitor) for 30 min or with $10^{-6}$M naloxone (a selective MOR inhibitor) for 2 h before LPS stimulation. In experiments involving two LPS pulses, cells were stimulated once with 10 μg/ml LPS for 24 h plus or not $10^{-6}$M EM-1, recollected by centrifugation, re-suspended in complete medium and then stimulated for further 24 h with 10 μg/ml LPS. At each time-point, supernatants were collected and 100 μl aliquots were added to 3 ml Optifluor and analyzed by a liquid scintillator counter.

*Measurement of Leukotriene $B_4$ and Prostaglandin $E_2$ release*

1x10$^6$ cells/well were challenged with 10 μg/ml LPS in the presence or absence of $10^{-6}$M EM-1 for 24 h, and

supernatants were collected by centrifugation at 1200 rpm for 5 min. Leukotriene $B_4$ ($LTB_4$) and Prostaglandin $E_2$ ($PGE_2$) levels in the medium were then assessed by enzyme immunoassay (EIA, Cayman Chemical) following the manufacturer's instructions. Briefly, 50 µl $LTB_4$ or $PGE_2$ standard or samples were added to the pre-coated mouse monoclonal anti-rabbit IgG macrotitre plates for $LTB_4$ or to the pre-coated goat polyclonal anti-mouse IgG macrotitre plates for $PGE_2$. Subsequently, 50 µl $LTB_4$ or $PGE_2$ tracer and 50 µl monoclonal $LTB_4$ or $PGE_2$ antiserum were added into each well and the mixture was incubated overnight at 4°C. After incubation, the content of each well was removed and the wells were washed 5 times with PBS buffer containing 0.05% Tween-20. An aliquot of 200 µl Ellman's reagent was added to each well, and the mixture was incubated for 2 h at room temperature with occasional shaking. The solution optical density (O.D.) was determined by a multi-well spectrophotometer (ELISA reader) at 405 nm. $LTB_4$ and $PGE_2$ concentration in each sample was calculated as the concentration corresponding to sample's O.D. values plotted on their respective standard built-in curves.

*Evaluation of ROI production by 2', 7'- dichlorofluorescein diacetate (DCF-DA) fluorescence*

$1x10^6$ cells were seeded in serum-free medium to prevent DCF-DA degradation by serum components and loaded with the fluorescent dye DCF-DA (10 µM) for 30 min at 37°C in the dark. Upon diffusion through the cell membrane, DCF-DA is hydrolyzed by intracellular esterases to non-fluorescent DCF, which is then rapidly oxidized to highly-fluorescent DCF in the presence of ROI. The DCF fluorescence intensity, thus, is proportional to the amount of intracellular ROI formed. After the incubation with the dye, cells were recollected and centrifuged for 5 min at 1200 rpm at room temperature. In experiments involving a single LPS pulse, the cells were re-suspended in serum-free medium and challenged with 10 µg/ml LPS for different experimental times (5 – 180 min) or with 10 µg/ml LPS plus different EM-1 concentrations ($10^{-6}$ – $10^{-12}$M) for 45 min. When requested, monocytes were incubated with $10^{-8}$M DPI for 30 min or with $10^{-6}$M naloxone for 2 h prior to LPS pulse. Additionally, cells were also incubated with 10 µg/ml LPS plus different EDTA concentrations (5 – 20 mM) for 45 min. In experiments involving a double LPS pulse, the cells were stimulated with 10 µg/ml LPS plus or not $10^{-6}$M EM-1 for 24 h prior to DCF-DA loading, as previously described. Cells were then stimulated with further 10 µg/ml LPS for 45 min. Cell fluorescence was measured under continuous magnetic stirring and controlled temperature (37°C) in a computer-aided Perkin-Elmer luminescence spectrometer (Model LS 50 b), with excitation wavelength set at 485 nm and emission wavelength set at 530 nm using 5 and 10 nm slits, respectively, for each light path.

*Evaluation of nitrite ($NO_2$) production by Griess reaction*

In experiments involving a single LPS stimulation, $3.5 \times 10^6$ cells/well were seeded in complete medium and pulsed with 10 µg/ml LPS in the presence or absence of EM-1 ($10^{-6}$M – $10^{-12}$M) for 24 h. In experiments involving a double LPS stimulation, $1.75 \times 10^6$ cells/well were seeded in complete medium and challenged with 10 µg/ml LPS plus or not $10^{-6}$M EM-1 for 24 h, recollected by centrifugation at 1200 rpm for 5 min, re-suspended in complete medium and stimulated for further 24 h with 10 µg/ml LPS. At each time-point, supernatants were collected, and nitrite ($NO_2$) levels in the medium were evaluated by mixing equal volumes of supernatans and Griess modified reagent in a macrotitre plate. When requested, cells were incubated with $10^{-8}$M DPI prior or with $10^{-6}$M naloxone for 2 h prior to LPS stimulation. Upon incubation for 30 min at room temperature, the presence of $NO_2$ in the samples determines the formation of a fluorescent dye sensible to stimulation with a laser beam set at 550 nm. $NO_2$ concentration in each sample was thus desumed by plotting the average O.D. on a standard curve prepared with sodium nitrite ($NaNO_2$, 0.43 – 65 µM) before each experiment, and each sample was divided by its relative control (arbitrary set to 100%) and then multiplied by 100 to obtain the relative increase.

*Peripheral monocytes purification and Dendritic Cells (DCs) generation*

Peripheral Blood Mononuclear Cells (PBMCs) were isolated from buffy-coats of healthy donors with ages ranging between 18 and 40 years, kindly provided by "La Sapienza" University Transfusion Service. Cells were separated by density gradient centrifugation using Lympholyte-H, and monocytes were then positively separated using anti-CD14-labeled magnetic beads (MACS) and re-suspended in RPMI-1640 supplemented with 10% foetal calf serum (FCS), L-glutamine (2 mM), gentamycin (10 µg/mL), Hepes buffer (10 mM), sodium pyruvate (0.1 M) and non-essential aminoacids (0.1 M). Monocytes were then washed and cultured with fresh complete medium for 5 days in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF, 200 U/ml) and IL-4 (10 ng/ml) and in the presence or absence of $10^{-6}$M EM-1 to generate immature DCs (imDCs). imDCs were finally incubated for further 48 h with 1 µg/ml LPS to induce final maturation according to Martino et al. (15).

*Fluorochrome-Activated Cell Sorter (FACS) Analysis*

The following monoclonal antibodies (mAbs), anti-

HLA class I, -HLA DR, -CD25, -CD80, -CD86 and -DC–SIGN were used to characterize DCs population. The cells were stained with either FITC-, PE- or APC-conjugated mAbs and analyzed using a FACScalibur running Cellquest Software (Becton Dickinson, Mountain View, CA, USA).

*Reagents*

RPMI-1640, FBS, glutamine, pyruvate, penicillin, streptomicin and sodium pyruvate were acquired from Cambrex. FCS was acquired from Hyclone. Lympholyte-H was acquired from Cederlane. Anti-CD14-MACS were acquired from Milteny Biothec. GM-CSF and IL-4 were acquired from Euroclone. Gentamycin, [$^3$H]-AA, LPS, DCF-DA, trypan blue, EM-1, non-essential aminoacids, Hepes buffer and Griess modified reagent were acquired from Sigma Aldrich. Monoclonal EIA kits for $LTB_4$ and $PGE_2$ were acquired from Cayman Chemicals. FITC-, PE- or APC-conjugated mAbs were acquired from Becton Dickinson Bioscience.

*Statistical analysis*

Data distribution was preliminarily verified by the Kolmogorov-Smirnov test, and data sets were analyzed by one-way analysis of variance (ANOVA), followed by Newman-Keuls test. Differences were regarded as significant when P-value was less than 0.05. Quantitative data were expressed as the percentile mean±S.D. of at least four replicate determinations each performed in triplicate, except where otherwise indicated.

## RESULTS

*Endomorphin-1 (EM-1) effects on the release of Arachidonic Acid (AA), Prostaglandin $E_2$ ($PGE_2$) and Leukotriene $B_4$ ($LTB_4$) from LPS-stimulated THP-1 monocytes*

Preliminary experiments were performed to evaluate THP-1 cells response to high LPS doses (1, 5 or 10 μg/ml) in terms of arachidonic acid (AA) release. All tested LPS doses determined a significant AA release (data not shown), with a maximum effect at 10 μg/ml which was then chosen as the dose for cell stimulation in all subsequent experiments. Moreover, since cytosolic phospholipase $A_2$ ($cPLA_2$) is one of the major enzymes involved in the mobilization of AA from membrane stocks to the extracellular spaces (16), experiments were performed to evaluate its possible involvement in LPS-stimulated AA release. The data shown in Fig. 1a show that, when cells were pretreated with MAFP

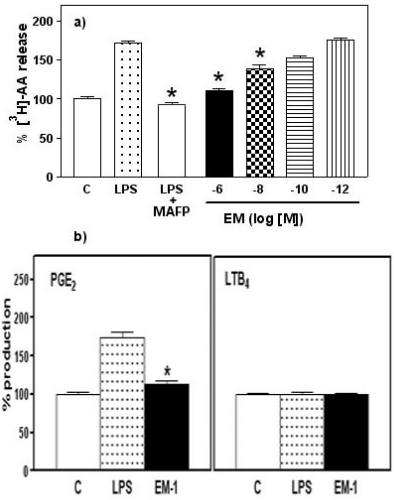


**Fig. 1.** *Endomorphin-1 (EM-1) effects on [$^3$H]-AA, $PGE_2$ and $LTB_4$ release from LPS-stimulated THP-1 cells. $1x10^6$ cells were:* ***a)*** *marked with 1 μCi [$^3$H]-arachidonic acid ([$^3$H]-AA) for 3 h, pretreated or not with 10 μM MAFP (a selective $cPLA_2$ inhibitor) for 30 min and incubated with 10 μg/ml LPS plus different EM-1 concentrations ($10^{-6}$–$10^{-12}$M) for 24 h, or* ***b)*** *incubated with 10 μg/ml LPS plus $10^{-6}$M EM-1 for 24 h. [$^3$H]-AA, $PGE_2$ and $LTB_4$ release were esteemed as reported in the Materials and Methods Section. Data are reported as the percentile mean ± SD of 4 independent experiments. *$p < 0.05$, as from ANOVA followed by Newman-Keuls test, in respect to LPS-treated cells (LPS)*

(a selective $cPLA_2$ inhibitor), LPS did not exert any significant effect, highlighting the involvement of this enzyme in LPS-stimulated AA release.

In order to evaluate possible EM-1 effects on LPS-stimulated AA release, cells were stimulated with LPS in the presence or absence of different EM-1 concentrations ($10^{-6}$ – $10^{-12}$M) for 24 h. Data shown in Fig. 1a demonstrate that the highest EM-1 concentrations ($10^{-6}$ and $10^{-8}$M) were able to significantly inhibit LPS-induced AA release, while lower concentrations were ineffective.

To evaluate possible EM-1 effects on the production of major arachidonic acid-derived

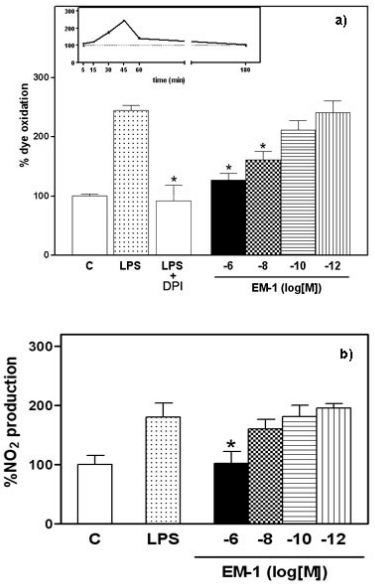


**Fig. 2.** *EM-1 effects on oxidant molecules production in LPS-stimulated THP-1 cells.* ***a)*** *1x10$^6$ cells were pretreated or not with 10$^{-8}$M DPI, a specific NADPH oxidase inhibitor), for 30 min and incubated with 10 µg/ml LPS plus different EM-1 concentrations (10$^{-6}$–10$^{-12}$M) for 45 min. Inset: 1x10$^6$ cells were incubated with 10 µg/ml LPS for different experimental times (5 – 180 minutes).* ***b)*** *3.5 x 10$^6$ cells were incubated with 10 µg/ml LPS plus different EM-1 concentrations (10$^{-6}$–10$^{-12}$M) for 24 h. ROI and $NO_2$ production was esteemed as reported in the Materials and Methods Section. Data are reported as the percentile mean ± SD of 4 independent experiments. *p < 0.05, as from ANOVA followed by Newman-Keuls test, in respect to LPS-treated cells (LPS)*

inflammatory factors, the levels of $PGE_2$ and $LTB_4$ in the medium were evaluated upon cell stimulation with LPS plus 10$^{-6}$M EM-1 for 24 h. The data shown in Fig. 1b demonstrate that LPS determined a significant increase in $PGE_2$ production, which was abrogated by the simultaneous incubation with the peptide. Conversely, both the incubation with LPS alone or with LPS + EM-1 did not exert any effect on $LTB_4$ levels, which remained equal to control.

*EM-1 suppresses LPS-stimulated oxidant molecules production in THP-1 monocytes*

To investigate the possible effects of EM-1 on LPS-induced ROI production, the cells were stimulated with LPS plus or not EM-1 (10$^{-6}$ – 10$^{-12}$M) for 45 min, a time-point at which a maximal ROI production occurs as demonstrated from preliminary time course experiments (5 min – 3 h) on ROI production kinetics shown in Fig. 2 inset. In addition, to assess a possible involvement of NADPH oxidase, a major enzyme involved in ROI production in phagocytes (17), experiments were performed pre-treating cells with DPI, a selective NADPH oxidase inhibitor. Data in Fig. 2a show that no significant ROI production could be observed in DPI-pretreated cells following LPS pulse, highlighting the involvement of NADPH oxidase in ROI production. Moreover, data in Fig. 2a also show that the highest EM-1 concentrations (10$^{-6}$ and 10$^{-8}$M) were able to significantly inhibit LPS-stimulated ROI production, while lower concentrations were ineffective.

In separate experiments, cells were also stimulated with LPS plus or not EM-1 (10$^{-6}$ – 10$^{-12}$M) for 24 h and $NO_2$ levels of in the medium were evaluated. Data shown in Fig. 2b demonstrate that only the highest EM-1 concentration (10$^{-6}$M) resulted in a significant inhibition of LPS-induced $NO_2$ production, while lower concentrations did not determine any significant effect.

*LPS-induced ROI production is $Ca^{2+}$-dependent and is required for both AA and $NO_2$ release from THP-1 monocytes*

Since it has been reported that NADPH oxidase-dependent ROI production can act as a signaling mechanism leading to $cPLA_2$ activation and $NO_2$ production (18-19), experiments were performed to evaluate whether LPS-induced release of AA and $NO_2$ was affected by NADPH oxidase inhibition. Data shown in Fig. 3 demonstrate that, when ROI production was inhibited by DPI, a) no AA release nor b) $NO_2$ production could be observed after LPS pulse, demonstrating that ROI production is a priming factor for the subsequent release of both AA

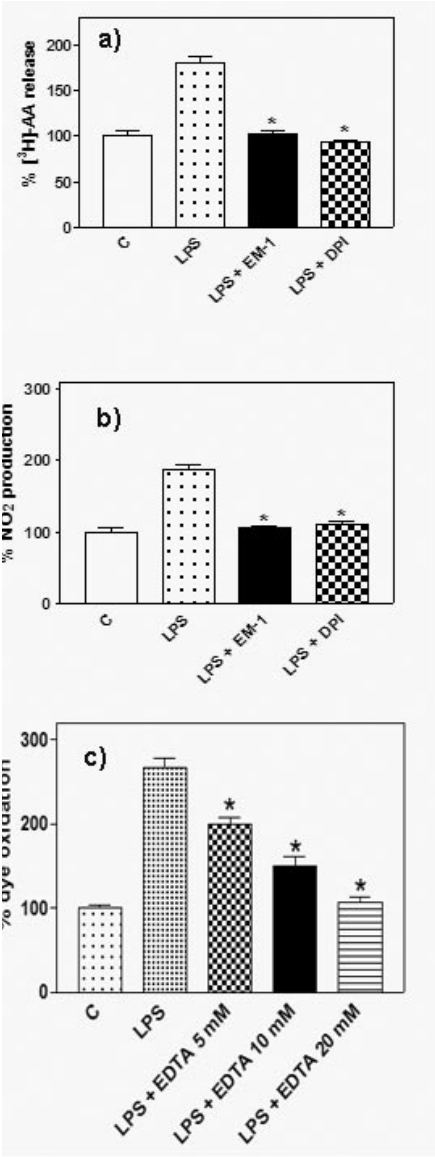


**Fig. 3.** *ROI production is $Ca^{2+}$-dependent and is required for both AA and $NO_2$ release in LPS-stimulated THP-1 cells.* ***a)*** *1 x $10^6$ cells for AA release experiments or* ***b)*** *3.5 x $10^6$ cells for $NO_2$ release experiments were pretreated or not with $10^{-8}$M DPI for 30 min and then incubated with 10 µg/ml LPS plus $10^{-6}$M EM-1for 24 h.* ***c)*** *1 x $10^6$ cells were incubated with 10 µg/ml LPS plus different EDTA concentrations (5-20 mM) for 45 min. ROI production and AA and $NO_2$ release was esteemed as reported in the Materials and Methods Section. Data are reported as the percentile mean ± SD of 4 independent experiments. *p < 0.05, as from ANOVA followed by Newman-Keuls test, in respect to LPS-treated cells (LPS)*

and $NO_2$. Moreover, since it has been reported that $Ca^{2+}$ is involved in LPS-induced ROI production (20), experiments were performed in the presence of different EDTA concentrations (5 – 20 mM) to deplete extracellular $Ca^{2+}$. Data in Fig. 3c show that LPS-induced ROI production was dose-dependently inhibited by EDTA, 20 mM being the most effective concentration, allowing us to hypothesize that $Ca^{2+}$ currents are a priming factor in LPS-triggered signaling mechanisms.

*EM-1 effects are mediated by µ opioid receptor (MOR) activation*

In order to evaluate whether EM-1 effects were mediated by µ opioid receptor (MOR), experiments were performed incubating cells with naloxone, a specific MOR inhibitor, before stimulation with LPS plus EM-1. Data in Fig. 4 show that naloxone pre-treatment blocked the inhibitory effect of $10^{-6}$M EM-1 on LPS-stimulated release of both AA and oxidant molecules, suggesting MOR involvement in EM-1 induced effects.

*EM-1 prevents the development of a hyporesponsive phenotype*

Monocyte hyporesponsivity triggered by repeated stimulations is a major pathological event characterizing the late phases of systemic inflammatory syndromes, which exposes the host to further aggressions (11). It would therefore be of key clinical importance to avoid such an aberrant derivation of monocyte functions (10). Since our previous data showed the efficacy of EM-1 administration in suppressing THP-1 cells

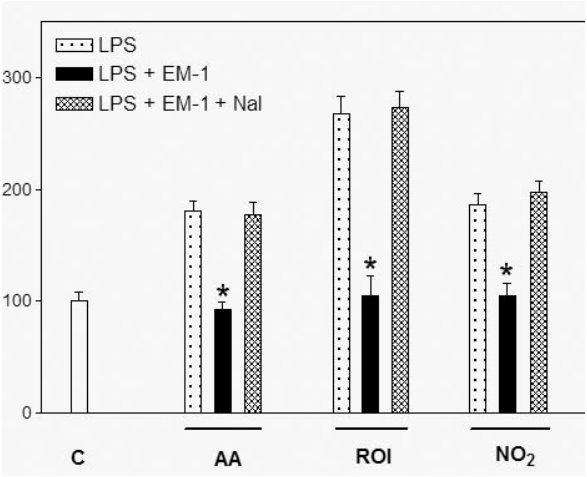


**Fig. 4.** *EM-1 effects are mediated by μ opioid receptor (MOR). 1 x $10^6$ cells in the experiments involving ROI production and AA release or 3.5 x $10^6$ cells in the experiments involving $NO_2$ release were pretreated or not with or with $10^{-6}$M naloxone (Nal, a selective MOR inhibitor) for 2 h prior to LPS stimulation for 45 min (ROI production) or 24 h (AA and $NO_2$ release) with or without $10^{-6}$M EM-1. ROI, AA and $NO_2$ were esteemed as reported in the Materials and Methods Section. Data are reported as the percentile mean ± SD of 4 independent experiments. *p < 0.05, as from ANOVA followed by Newman-Keuls test, in respect to LPS-treated cells (LPS)*

activation, we have developed a model of monocyte hyporesponsivity induced by a double overnight pulse with high LPS doses, and tested possible EM-1 effects in preventing the establishment of such a phenotype. Data shown in Fig. 5 demonstrate that a double LPS pulse (LPS+LPS) results in the absence of both AA release (a) and ROI production (b) in respect to the cells stimulated with a single LPS pulse (LPS), while an abnormal increase in $NO_2$, whose levels were higher than the ones characterizing single LPS-stimulated monocytes, could be observed (c).

Basing on these results, then, we tested whether the addition of $10^{-6}$M EM-1 during the first LPS pulse could re-establish monocyte responses to the second pulse. Data shown in Fig. 5 demonstrate that the addition of EM-1 allows monocytes to preserve their ability to respond to the second LPS pulse (LPS+EM-1+LPS), as demonstrated by a restored AA release (a) and ROI production (b), with a concomitant decrease in $NO_2$ levels which tended to be equal to the ones characterizing single LPS-stimulated monocytes (c).

*EM-1 effects on dendritic cell (DC) generation*

Since it has been recently reported that an alteration in monocyte inflammatory metabolism plays a key role in controlling both the physiological and the pathological development to DCs (10-13), experiments have been performed to evaluate possible EM-1 interference on peripheral monocyte development to DCs in terms of the expression of specific transductional (CD25, DC-SIGN), co-stimulatory (CD80, CD86) and immuno-stimulatory (HLA-I, HLA-DR) markers. Data in Fig. 6 show that the addition of $10^{-6}$M EM-1 to developing DCs does not significantly alter the expression of any chosen marker.

## DISCUSSION

Endomorphin-1 (EM-1) is an endogenous peptide behaving as an opiate drug on the nervous

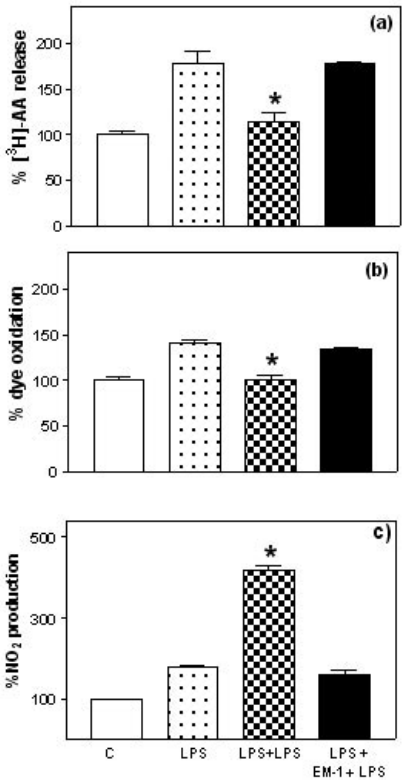


**Fig. 5.** *EM-1 effects on the development of the hyporesponsive phenotype. Cells were incubated with 10 μg/ml LPS plus $10^{-6}$M EM-1 for 24 h, washed and re-stimulated with 10 μg/ml LPS and assays were performed as reported in the Materials and Methods Section. Data are reported as the percentile mean ± SD of 4 independent experiments. *p<0.05, ANOVA followed by Newman-Keuls test, in respect to LPS-stimulated cells (LPS)*

system (1), being mainly endowed with endogenous analgesia (antinociception) by means of its ability to bind to μ opioid receptors (MORs) (21). Coherently, EM-1 has been traced in those areas of the brain and the central nervous system (CNS) which have a regulatory activity on the body pain perception (2, 21). Interestingly, outside the CNS, the immune system is a major EM-1 producing site: different leucocyte subpopulations, in fact, have been demonstrated to actively produce endomorphins, which in turn could contribute to decrease the sensory neuron firing rate (22-23). Moreover, leucocytes have been also demonstrated to express MORs, thus allowing to hypothesize that EM-1 could exert multiple analgesic/anti-inflammatory effects in both an autocrine and paracrine fashion (22-24). Notably, recent evidence demonstrated that EM-1 can actively cross the blood-brain barrier (BBB), thus diffusing into the blood and possibly reaching distant inflamed tissues (4-5), also affecting the functions of circulating MOR-expressing cells.

In this context, however, it still lacks any knowledge about EM-1effects on circulating cells belonging to the systemic compartment of the immune response, such as monocytes. Monocytes, in fact, represent the first defense line against invading pathogens, and their ability to release a vast range of pro-inflammatory molecules in the blood flow is a major event in the induction of systemic reactions (8-9). However, monocytes activated by an excessive pathogenic stimulus release huge amounts of inflammatory molecules leading to life-threatening multiple organ failure (9). Moreover, a generalized immunosuppressed state (a condition usually referred to as hyporesponsivity), generally establishes itself after an intense inflammatory response (11). Such a also for the host, and it is mainly believed to depend on monocyte hyporesponsivity or on an altered monocyte differentiation to DC (10-11, 13).

On the basis of such evidence, therefore, the aim of this work is to evaluate possible EM-1 immunomodulatory effects on a model of intense monocyte activation consisting of THP-1 cells challenged with high LPS doses, in an attempt to recreate *in vitro* a situation occurring *in vivo* during septic states. The use of a reliable human monocyte model (25) in this experimental framework allowed us to avoid the inter- and intra-specific variability which usually arises in human or animal peripheral blood mononuclear cells (PBMCs) (26), thus facilitating the interpretation of extrapolated data.

In this context, our data prove for the first time that EM-1 exerts a significant inhibitory activity on monocytes, for it suppressed the production of chosen activation markers, comprising ROI,

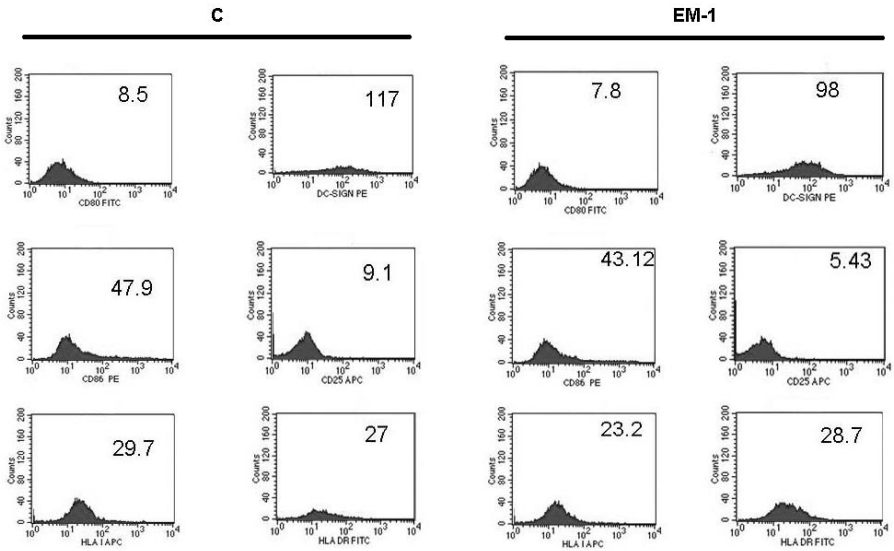


**Fig. 6.** ***EM-1 effects on DCs development.*** *1x10$^6$ cells were incubated with 200 U/ml GM-CSF and 10 ng/ml IL-4 plus or minus 10$^{-6}$M EM-1 for 5 days, and FACS analysis was performed as reported in the Materials and Methods Section. A significant experiment is shown, which was repeated two additional times with similar results.*

$NO_2$, AA and $PGE_2$. Notably, other evidence seems to be of particular interest: the use of high LPS doses, in fact, resulted in the alteration of monocyte AA metabolism, which was shifted from an inflammatory (i.e., $LTB_4$-producing) profile to an immunosuppressive (i.e., $PGE_2$-producing) one. Intriguingly, these observations provide both good feedback on our experimental model, for they resemble recent data obtained from macrophages endowed with systemic inflammatory syndromes and immunosuppression (27), and also possibly explain such a phenomenon as a consequence of a previously-altered monocytic state.

Basing on these observations, we subsequently tested whether, in our experimental model, hyporesponsivity could be observed after sequential overnight incubation of these cells with two high doses of LPS pulses. Interestingly, as a response to such a protocol, cells did not respond to the second pulse in terms of both ROI production or AA release, while an abnormal $NO_2$ production, which is considered to be immunosuppressive (27-28), was observed. In such a simple desensitization model, EM-1 proved to block both AA release and ROI production, concomitantly normalizing $NO_2$ production.

Interestingly, considering the immunosuppressive effects of excessive $NO_2$ production (27-28), our data suggest that EM-1 could modulate monocyte responsivity through the modulation of $NO_2$ levels. Furthermore, our data provide the first evidence of the mechanism by which EM-1 could exert its effects on LPS-stimulated cells. We demonstrate, in fact, that LPS induces a rapid NADPH oxidase-dependent ROI production, which in turn activates both cytosolic $PLA_2$ ($cPLA_2$)-dependent AA release and $NO_2$ production. The whole pathway seems to be $Ca^{2+}$-dependent, since no ROI production

could be observed when the cells were pulsed with LPS in the presence of EDTA. In this context, the observed ability of EM-1 to suppress LPS signaling cascade could be easily linked to a decrease in the intracellular $Ca^{2+}$ content, since it has already been demonstrated that MOR activation is coupled to calcium current inhibition (29).

Finally, since any alteration in DCs is likely to deregulate the inflammatory reactions and to spread immunosuppression (30), we analyzed EM-1 effects on DC showing that, when the peptide was added together with GM-CSF and IL-4 to developing DCs, no significant effects could be observed on their specific receptor set. These observations suggest that EM-1 immunosuppressive activities are not extended to DC development, thus providing the evidence that EM-1 should not hamper the development of specific immune responses.

Taken together, our data represent the first characterization of EM-1 effects on monocytes, also providing clues for a possible EM-1 clinical application in inflammatory pathologies and as a useful combination to an antibiotic prophylaxis in septic states.

## ACKNOWLEDGEMENTS

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# Brain Natriuretic Peptide (BNP) regulates the production of inflammatory mediators in human THP-1 macrophages

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ABSTRACT

Brain Natriuretic Peptide (BNP), besides retaining vasodilatory, diuretic and natriuretic properties, is a vasoactive hormone that it is also involved in several cardiac diseases as well as severe sepsis and septic shock. All these conditions are characterized by an ongoing inflammatory response consisting in a complex interaction of pleiotropic mediators derived from plasma or cells, including monocytes and macrophages. However, the relationship between this hormone and inflammation remains to be elucidated. Therefore, the aim of the present study was to evaluate a possible BNP immunomodulatory activity on macrophages. Our results demonstrate that BNP regulates the production of major inflammatory molecules, such as reactive oxygen- and nitrogen species (ROS and RNS), leukotriene $B_4$ ($LTB_4$), prostaglandin $E_2$ ($PGE_2$); modulates the cytokines (TNF-α, IL-12 and IL-10) profile, and affects cell motility. These results furnish novel and brand-new proofs on BNP ability of modulating the production of inflammatory mediators in macrophages whose role has broad implications in inflammatory states where increased BNP levels have been reported.



## 1. Introduction

Brain Natriuretic Peptide (BNP), a member of the natriuretic peptides (NPs) family, which also includes Atrial Natriuretic Peptide (ANP) and C-type Natriuretic Peptide (CNP), is a low molecular weight peptide mainly involved in the regulation of heart and renal homeostasis as well as in the modulation of vascular cell functions [1]. The effects of BNP in the cardiovascular system have been widely studied and investigations have mainly focused on the diuretic, natriuretic, and vasodilating properties of the peptide [2].

However, it is increasingly recognized that the peptide functions are not only restricted to the regulation of volume homeostasis, but BNP and its receptors are also expressed in diverse tissues besides the cardiovascular and renal system [3]. In particular, BNP has been recently linked to the immune system, thus providing new aspects of the biological profile of this natriuretic peptide. Many evidence about possible BNP immunomodulatory activities are currently emerging. In this context, BNP and N-terminal pro-BNP levels are highly elevated in patients with severe sepsis or septic shock [4] owing to either increased secretion or decreased degradation induced by inflammation [5,6]. Accordingly, results from animal studies and tissue cultures show that both the production and secretion of the natriuretic peptide are activated by endotoxin and inflammatory mediators [7,8]. Furthermore, Vollmar et al. [9] found that the natriuretic peptides are expressed and differentially regulated in thymus, spleen, lymph nodes, tonsils, as well as in specialised immune cells such as macrophages [10], suggesting a role for BNP during inflammation. Indeed, macrophages represent a functionally heterogeneous population of immune cells that hold a crucial role in inflammatory processes [11]. In response to an immune challenge, macrophages are activated and migrate or produce a wide array of proinflammatory mediators that contribute to non-specific immunity [11]. Increased BNP levels have been found in several inflammatory pathologies, but no investigations have been carried out about its possible effects on macrophage activation. Hence, the aim of the present study was to investigate the possible effects of BNP on the major macrophage inflammatory activities, such as reactive oxygen and nitrogen species (ROS and RNS, respectively) production, arachidonic acid metabolites ($LTB_4$, $PGE_2$) and cytokines (TNF-α, IL-12 and IL-10) release. Furthermore, the macrophage migratory activity was also evaluated. Our findings display a first evidence of BNP effect on macrophages activation, thus providing novel insights to understand the regulation of the inflammatory network in different pathophysiological scenarios.

## 2. Materials and methods

### 2.1. Cell cultures and treatments

The human monocytic cell line THP-1 was obtained from the American Type Culture Collection (ATCC, Rockville, MD) and cultured in

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Falcon flasks with RPMI-1640 medium supplemented with 10% Foetal Bovine Serum (FBS), L-glutamine (2 mM), sodium pyruvate (100 μg/ml), penicillin (100 U/ml) and streptomycin (100 μg/ml). Cells were cultured in a humidified incubator at 37 °C in a 5% $CO_2$ atmosphere. THP-1 macrophages (MΦ) were obtained incubating monocytes with $10^{-6}$ M phorbol 12-myristate 13-acetate (PMA) for 72 h. Cell viability was routinely assessed by Trypan blue exclusion method using a Neubauer modified chamber.

### 2.2. Evaluation of ROS production by 2′,7′-dichlorofluorescein diacetate (DCF-DA) fluorescence

$1 \times 10^6$ MΦ/well were incubated in the dark with 10 μM 2′,7′-dichlorofluorescein diacetate (DCF-DA) in serum-free medium for 30 min at 37 °C. DCF-DA diffuses through the cell membrane and is hydrolyzed by intracellular esterases to nonfluorescent DCF deacetylated, which is then rapidly oxidized to highly fluorescent DCF in the presence of ROS. The DCF fluorescence intensity is proportional to the amount of intracellular ROS formed [12]. After the incubation with the fluorescent dye, cells were then harvested and the medium containing the unincorporated dye was eliminated by centrifugation. Cells were then stimulated in serum-free medium with different BNP concentrations ($10^{-8}$–$10^{-10}$ M) for different experimental times (3–9 h). In those experiments involving diphenylene iodinium (DPI), a NADPH oxidase inhibitor, cells were incubated with $10^{-8}$ M DPI for 30 min before BNP addition. At each timepoint, cells were harvested, resuspended in phosphate buffered-saline (PBS), and assessed for changes in fluorescence using a background-controlling computer-aided LS-50b fluorescence spectrometer (Perkin Elmer) with excitation wavelength set at 485 nm and emission wavelength set at 530 nm, using 5 and 10 nm slits for each light path, respectively. Results were expressed as fluorescence intensity, reported as Fluorescence Units (F.U.), in respect to cells loaded with only DCF-DA (C).

### 2.3. Evaluation of nitrite ($NO_2$) production by Griess reaction

$1 \times 10^6$ MΦ/well were challenged with $10^{-9}$ M BNP for 3, 6, 9 h and up to 24 h and, at the end of the experimental times, supernatants were collected and nitrite ($NO_2$) levels in the medium were determined by a colorimetric assay. Briefly, each sample was probed in triplicate by adding 100 μl of Griess Reagent (modified) to 100 μl aliquots obtained from each of the samples in a 96-well macrotitre plate. Wells were mixed repeatedly and the reaction was performed at room temperature for 30 min. At the end of this time, the absorbance of each well was read by means of an EIA reader 450 (Biorad) with wavelength set at 550 nm. $NO_2$ concentration in each sample was calculated as the concentration corresponding to samples' optical density (O.D.) values plotted on a standard curve routinely prepared for each experiment using sodium nitrite ($NaNO_2$, 0,43–65 μM) as the reference. Values were then divided by their respective controls and multiplied by 100 to obtain the percentile increase.

### 2.4. [$^3$H]-arachidonic acid ([$^3$H]-AA) release assay

Preliminary experiments on [$^3$H]-arachidonic acid ([$^3$H]-AA) incorporation indicated that optimal radioactive labelling was obtained incubating cells with 1 μCi of [$^3$H]-AA (specific activity: 202,4 Ci/mmol) for 3 h [13]. $1 \times 10^6$ MΦ/well were then incubated for 3, 6 and 9 h with 1 μCi [$^3$H]-AA in serum-free medium. To remove non-specific binding of [$^3$H]-AA to cell surface prior to agonist stimulation, culture medium was eliminated, cells resuspended in serum-free medium and challenged with $10^{-9}$ M BNP for the different experimental times. In those experiments involving methyl arachydonil fluorophosphate (MAFP, a selective phospholipase $A_2$ cytosolic ($cPLA_2$) inhibitor), cells were treated with 10 μM MAFP for 30 min before BNP stimulation. After each treatment, conditioned medium was collected by centrifugation and 100 μl aliquots were added to 3 ml Optifluor and analyzed in a Tricarb 2180 TR liquid scintillation counter (Packard Instruments). Results were reported as percentile increase with respect to controls (C).

### 2.5. Leukotriene $B_4$ ($LTB_4$) and prostaglandin $E_2$ ($PGE_2$) release assay

$1 \times 10^6$ MΦ/well were pre-treated or not with 10 μM MAFP for 30 min before challenging with $10^{-9}$ M BNP for 3 h and, at the end of experimental time, supernatants were collected and leukotriene $B_4$ ($LTB_4$) and prostaglandin $E_2$ ($PGE_2$) levels in the medium were determined by enzyme immunoassay (EIA) (Cayman Chemical), following manufacturer's instructions. Briefly, 50 μl $LTB_4$ standard or samples were added to the pre-coated mouse monoclonal anti-rabbit IgG macrotitre plates. Subsequently, 50 μl $LTB_4$ tracer and 50 μl monoclonal antiserum of $LTB_4$ were added into each well and the mixture was incubated overnight at 4 °C. After incubation, the content in each well was removed and the wells were washed 5 times with PBS buffer containing 0.05% Tween-20. An aliquot of 200 μl Ellman's reagent was added into each well, and the mixture was incubated for 2 h at room temperature with occasional shaking. The O.D. of the solution was determined by a multi-well spectrophotometer (ELISA reader) at 405 nm. A similar procedure was performed for $PGE_2$ release determination, using pre-coated goat polyclonal anti-mouse IgG macrotitre plates. $LTB_4$ and $PGE_2$ concentration in each sample was calculated as the concentration corresponding to sample's O.D. values plotted on respective standard built-in curves. Values were reported as percentile increase with respect to controls (C).

### 2.6. Cytokines production assay

The interleukins 10, 12, and TNF-α were determined by microplate ELISA using commercially available appropriate kits from Pierce Endogen according to the procedure recommended by the manufacturer. Briefly, $1 \times 10^6$ MΦ/well were challenged with $10^{-9}$ M BNP for 24 h and supernatants were then collected and eluted. The absorbance of each well was measured by ELISA reader with wavelength set at 450 nm. Cytokines concentrations in each sample was calculated as the concentration corresponding to samples' O.D. values plotted on a standard built-in curve and then reported as percentage values.

### 2.7. Wound healing assay

$1 \times 10^6$ monocytes/ml were plated in a chamber slide (Labtek, Queensland, Australia) with RPMI-1640 medium containing 10% FBS and $10^{-6}$ M PMA for 72 h to obtain THP-1MΦ. The cells were grown to confluence and the monolayers were then wounded with a 10 μl pipette as described [14]. After wounding, cells were washed three times with PBS 1× to remove suspended cells and incubated for 24 h in serum-free medium with or without $10^{-9}$ M BNP. Subsequently, the cells were fixed with paraformaldehyde 4% in PBS 1× and labelled for F-actin using tetramethylrhodamine isothiocyanate (TRITC)-conjugated phalloidin. Nuclei were stained with 4′,6′-diamidino-2-phenylindole (DAPI). Images of cell samples were taken with a Leica DMRB microscope using a digital camera. The images were representative of five random fields in two slide preparations for each sample.

### 2.8. Reagents

RPMI-1640, L-glutamine, penicillin, and streptomycin were from Eurobio Laboratoires. Foetal Bovine Serum (FBS) was from GIBCO (Grand Island NY, USA). Human Brain Natriuretic Peptide-32 (BNP), Trypan blue, DPI, MAFP, DCF-DA, PMA, sodium pyruvate, DAPI and paraformaldehyde were from Sigma-Aldrich, Milano, Italy. [$^3$H]-arachidonic acid (202,4 Ci/mmol) from Amersham Biosciences, NJ, USA. Tetramethylrhodamine isothiocyanate (TRITC)-conjugated phalloidin from Invitrogen Corp, CA,

USA. Leukotriene $B_4$ and prostaglandin $E_2$ assay kits were purchased from Cayman Chemical. IL-10, IL-12 and TNF-α assay kits were from Pierce Endogen. All reagents, especially mediums, BNP and PMA, were routinely and accurately checked for any endotoxin contamination by means of Limulus amebocyte lysate test (Bio Whittaker, Walkersville, MD).

### 2.9. Statistical analysis

Data distribution was preliminarily verified by the Kolmogorov–Smirnov test, and data sets were analyzed by one-way analysis of variance (ANOVA) followed by Student–Newman–Keuls (SNK) test. Differences were regarded as significant when P value was less than 0.05. Quantitative data were expressed as the percentile mean ± S.D. of at least four replicate determinations each performed in triplicate, except where otherwise indicated.

## 3. Results

### 3.1. BNP effect on reactive oxygen species (ROS) and nitrite ($NO_2$) production in THP-1 macrophages (Mϕ)

In order to investigate whether BNP could regulate reactive oxygen species (ROS) and nitrite ($NO_2$) production in human THP-1 macrophages (Mϕ), different experiments were performed. At first, to evaluate BNP ability to stimulate ROS production, cells were incubated with 10 μM DCF-DA and then challenged with a range of hormone concentrations ($10^{-8}$–$10^{-10}$ M), varying from physiological to pharmacological ones, for different experimental times (3, 6 and 9 h). The dose response curve and time course of the BNP effect on ROS production in THP-1Mϕ are reported in Fig. 1. In this regard, BNP exerts significant effects on ROS production after 3 h of treatment independently of the concentration tested, showing a maximal effect at $10^{-9}$ M BNP concentration, while no significant effects were detectable at longer experimental times (6 and 9 h). For this reason, in all experiments to be reported, BNP effects were evaluated challenging THP-1Mϕ with $10^{-9}$ M BNP. The relatively high background fluorescence found in untreated cells is definitely related to the treatment with PMA.

Since NADPH oxidase is the main enzyme complex implicated in ROS production in phagocytic cells [15], we assessed whether it could be involved in such BNP-induced production. For this purpose, THP-1Mϕ labelled with 10 μM DCF-DA were incubated with $10^{-8}$ M of DPI, a selective NADPH oxidase inhibitor, and then challenged with $10^{-9}$ M BNP for 3 h. Data reported in Fig. 2 show that BNP-induced ROS production is dampened by the pre-treatment with the inhibitor and that DPI alone does not affect ROS production. These results suggest that BNP can exert a significant effect on ROS production in THP-1Mϕ through the involvement of NADPH oxidase.

Later on, in order to furnish a further evaluation of BNP capability to affect the release of oxidant molecules, experiments were performed to evaluate whether this hormone could induce $NO_2$ production. Since there is evidence for a shift in the time at which maximal ROS and RNS production can occur [16], such evaluation was assessed at 3, 6, 9 h and up to 24 h. Fig. 3 shows that $10^{-9}$ M BNP induces a significant increase in $NO_2$ production which is already detectable at 3 h, reaches a maximum at 6 h and maintains a plateau

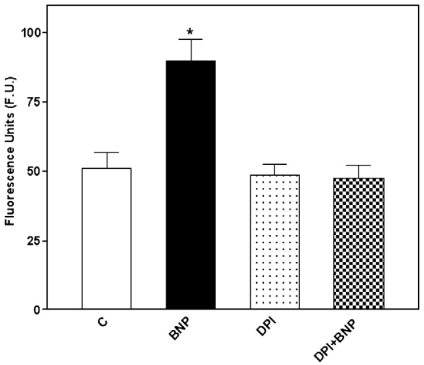


**Fig. 2.** BNP effect on ROS production in the presence of DPI in THP-1Mϕ. $1 \times 10^6$/ml cells were labelled with 10 μM DCF-DA, pre-treated with $10^{-8}$ M DPI for 30 min and then challenged with $10^{-9}$ M BNP for 3 h. ROS production was assessed as reported in the Materials and methods section and expressed as fluorescence intensity, reported as Fluorescence Units (F.U.), in respect to cells loaded with DCF-DA only (C). Data are reported as mean ± S.D. of 4 different experiments. *$p < 0.05$, as reported from SNK test in respect to unstimulated cells (C).

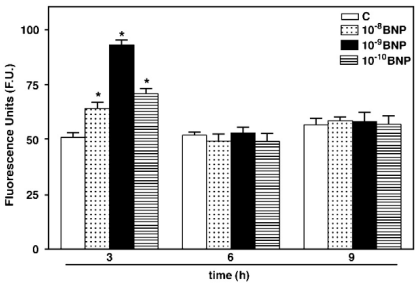


**Fig. 1.** BNP effect on ROS production in THP-1 macrophages (Mϕ). $1 \times 10^6$/ml cells were labelled with 10 μM DCF-DA, as reported in the Materials and methods section, and ROS production was assessed after cell exposure to different BNP concentrations ($10^{-8}$–$10^{-10}$ M) for 3, 6 and 9 h. Results are expressed as fluorescence intensity, reported as Fluorescence Units (F.U.), in respect to cells loaded with DCF-DA only (C). Data are reported as mean ± S.D. of 4 different experiments. *$p < 0.05$, as reported from SNK test in respect to unstimulated cells (C).

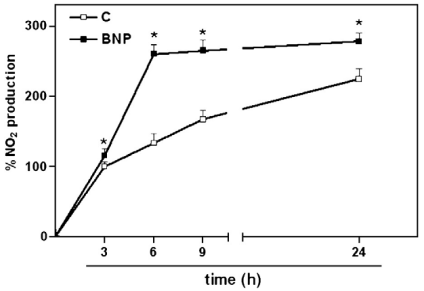


**Fig. 3.** BNP effect on $NO_2$ production in THP-1Mϕ. $1 \times 10^6$/ml cells were challenged with $10^{-9}$ M BNP for 3, 6, 9 h and up to 24 h. $NO_2$ released into the medium was assessed as reported in the Materials and methods section. Values, expressed as the optical density (O.D.) corresponding to $NO_2$ concentration per $1 \times 10^6$ cells, were divided by their respective controls and multiplied by 100 to obtain the percentile increase. Data are reported as percentile mean ± S.D. of 4 different experiments, whereas 100% of $NO_2$ production is 5 μM/$1 \times 10^6$ cells. *$p < 0.05$, as reported from SNK test in respect to unstimulated cells (C).

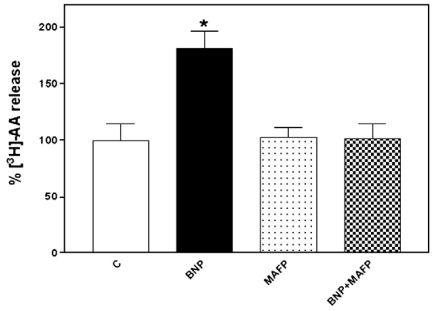


**Fig. 4.** BNP effect on arachidonic acid (AA) release in THP-1Mϕ. $1 \times 10^6$/ml cells were incubated for 3 h with 1 μCi/ml [$^3$H]-AA. After this time, culture medium was eliminated, cells resuspended in serum-free medium and challenged with $10^{-9}$ M BNP for 3 h. When requested, 10 μM MAFP was added for 30 min before BNP administration. The [$^3$H]-AA released into the medium was determined as reported in Materials and methods section. Data are reported as percentile mean ± S.D. of 4 different experiments. *$p < 0.05$, as reported from SNK test in respect to unstimulated cells (C).

up to 24 h of stimulation. As already stated, the high level of $NO_2$ production in controls is likely to be the result of treatment with PMA, especially after 24 h.

### 3.2. BNP stimulates the release of arachidonic acid (AA) and its metabolites

An important marker of macrophage activation is represented by arachidonic acid (AA) release from membrane phospholipids [17] which can partly depend on oxidative stress, leading to the activation of cytosolic phospholipase $A_2$ ($cPLA_2$), the main enzyme entailed in such release [18]. In this regard, to determine whether BNP could exert any effect on AA release, experiments were performed labelling THP-1Mϕ with 1 μCi [$^3$H]-AA and then challenging them with $10^{-9}$ M BNP in the presence or absence of 10 μM MAFP, a selective $cPLA_2$ inhibitor. Fig. 4 shows that BNP induces a significant AA release in respect to control cells after 3 h of stimulation, while no significant effects were detectable after 6 or 9 h of treatment (data not shown). Moreover, this process occurs through the involvement of $cPLA_2$ as the pre-treatment with MAFP was able to totally abolish BNP-induced AA release.

Arachidonic acid is a precursor of a plethora of immune active lipids, including prostaglandins and leukotrienes, which are the most represented molecules in activated macrophages [19]. In this regard, we investigated whether BNP could be able to affect leukotriene $B_4$ ($LTB_4$) and prostaglandin $E_2$ ($PGE_2$) production. Fig. 5 shows that BNP significantly activates both $LTB_4$ and $PGE_2$ release from THP-1Mϕ in respect to untreated cells. Moreover, such release is also $cPLA_2$-dependent because cells pre-treated with MAFP were totally unable to release both AA-derived metabolites. Taken together, these results suggest that BNP stimulates arachidonic acid release as well as $LTB_4$ and $PGE_2$ production through the involvement of $cPLA_2$.

### 3.3. BNP effects on cytokine production

Another hallmark of activated macrophage is represented by the release of a broad array of cytokines [11]. All cytokines display pleiotropic activities, yet TNF-α and IL-12 are mainly considered proinflammatory cytokines while IL-10 is a typical anti-inflammatory cytokine, being also able to inhibit the synthesis of the same pro-inflammatory ones [20]. Thus we investigated if BNP could, somehow, also be able to affect or regulate these cytokines release. Fig. 6

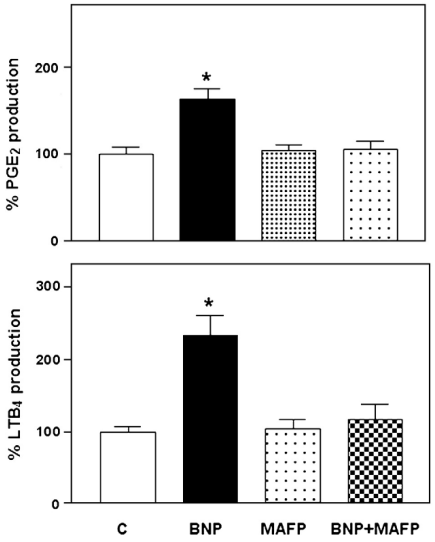


**Fig. 5.** BNP effect on leukotriene $B_4$ ($LTB_4$) and prostaglandin $E_2$ ($PGE_2$) release in THP-1Mϕ. $1 \times 10^6$/ml cells were challenged with $10^{-9}$ M BNP for 3 h. When requested, 10 μM MAFP was added for 30 min before BNP stimulation. $LTB_4$ and $PGE_2$ released into the medium were measured by EIA assays, as described in the Materials and methods section. Data are reported as percentile mean ± S.D. of 4 different experiments, whereas 100% of $LTB_4$ is 18 pg/$1 \times 10^6$ cells while 100% of $PGE_2$ is 20 pg/$1 \times 10^6$cells. *$p < 0.05$, as reported from SNK test in respect to unstimulated cells (C).

shows that BNP induces IL-10 release from THP-1Mϕ after 24 h of stimulation, while no variation can be detected in IL-12 and TNF-α release in respect to unstimulated cells.

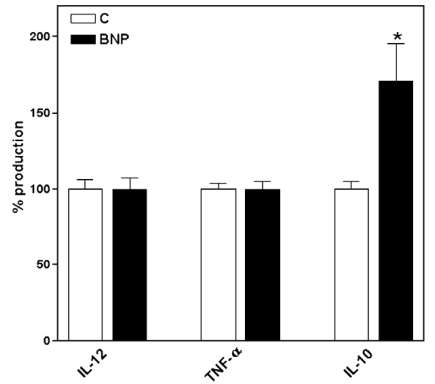


**Fig. 6.** BNP effect on cytokines release in THP-1Mϕ. $1 \times 10^6$/ml cells were challenged with $10^{-9}$ M BNP for 24 h and IL-12, TNF-α, and IL-10 released into the medium were measured by ELISA assays, as described in the Materials and methods section. Values, expressed in terms of pg/$1 \times 10^6$ cells, were divided by their respective controls and multiplied by 100 to obtain the percentile increase, whereas 100% of IL-12, TNF-α and IL-10 is 25.6, 14.8 and 13.8 pg/$1 \times 10^6$ cells, respectively. Data are reported as percentile mean ± S.D. of 4 different experiments. *$p < 0.05$, as reported from SNK test in respect to unstimulated cells (C).

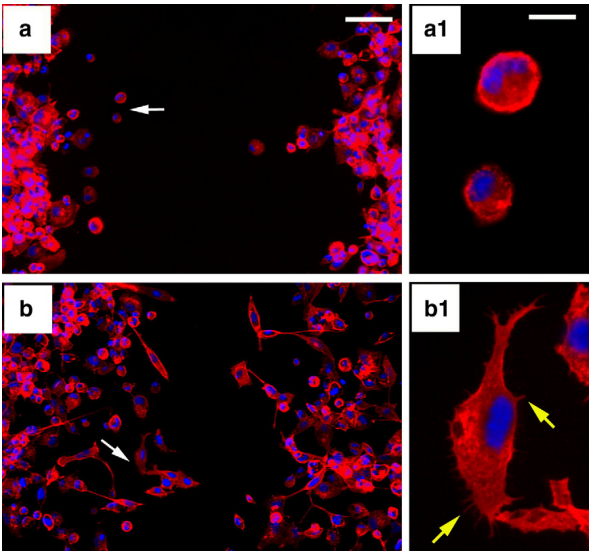


**Fig. 7.** BNP effect on THP-1Mϕ migratory capability. A confluent monolayer of THP-1Mϕ was 'wounded' with a pipette tip and incubated for 24 h in the absence (a) or presence of $10^{-9}$ M BNP (b). At the end of the treatment, cells were fixed with 4% paraformaldehyde and stained for polymerized actin (F-actin, in red) with tetramethylrhodamine isothiocyanate (TRITC)-conjugated phalloidin. At right of panel (a1 and b1) is showed a magnification of cells indicated by white arrows in a and b, respectively. In fig. b1, the yellow arrows indicate microspikes protruding from cell surface of THP-1Mϕ after $10^{-9}$ M BNP incubation. Nuclei were stained with DAPI (blue). Scale bars, 100 μm (a, b); 25 μm (a1, b1) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

### *3.4. BNP and THP-1Mϕ migratory capability*

During inflammatory processes, macrophages are essential cellular regulators that are recruited in inflamed sites and herein exert their multiple functions [21]. Thus, we assessed whether BNP could be able to affect THP-1Mϕ migratory capability. A wound healing assay, which is generally considered a simple and reliable test for evaluation of cell motility, was thus carried out. As it can be seen in Fig. 7, after 24 h of $10^{-9}$ M BNP treatment, THP-1Mϕ display clear healing and morphological changes (Fig. 7b) in respect to unstimulated cells (Fig. 7a), which are indicative of cell motility. At the molecular level, BNP-dependent migration was accompanied by cytoskeleton remodelling. Indeed, treated macrophages displayed typical F-actin rich microspikes (Fig. 7b1) in respect to unstimulated cells (Fig. 7a1), thus demonstrating BNP aptitude in regulating THP-1Mϕ migratory capability.

## 4. Discussion

Since its identification, BNP has been regarded as a prominent factor in renal and cardiovascular homeostasis [1]. Increased levels of BNP are considered a useful and a substantial predictive marker of several cardiac diseases, including myocardial infarction, congestive heart failure and coronary syndromes [5]. Moreover, much investigations of BNP effects on the activation of fibroblasts, endothelial and vascular smooth muscle cells, all of them being involved in those processes implicated in pathologies progression, has been extensively carried out [22,23]. Recently, evidence of an immunomodulatory role of BNP has been accumulated [5,6] but no information is presently available about possible effects of this hormone on leukocytes. Therefore, the present study aims then at investigating BNP effects on the activation of macrophages, whose role is crucial in those pathologies characterized by an ongoing inflammatory process.

The present results provided evidence that BNP is able to extensively stimulate ROS production through NADPH oxidase and to significantly increase $NO_2$ release in THP-1 macrophages. The involvement of NADPH oxidase, one of the major macrophage ROS-producing enzyme complex [15], can be envisioned even though the involvement of several other ROS-producing flavin-dependent enzymes, cannot be excluded [24,25]. Although necessary for adequate immunity, ROS and RNS produced in high amounts by activated macrophages may cause damage in host cells and contribute to sustain the pathogenesis of several inflammatory diseases, such as septic shock or arthritis [26]. Therefore, unraveling the mechanisms regulating ROS and RNS production is of particular interest to understand the pathogenesis of several diseases. In this context, the role of BNP might be relevant also considering that myocardial damage related to an ischemic event is characterized by an important inflammatory component [27]. Furthermore, it is now well recognized that ROS and RNS may interact with each other, resulting both in the induction of further new reactive species and in possible changes in their time of occurrence [26]. In this regard, our different kinetics of maximal ROS and $NO_2$ production obtained at 3 h and 6 h of stimulation, respectively, may represent an amenable validation for such process, above all concerning the loss of response of ROS production at 6 h and 9 h of BNP stimulation, but it could also suggest that BNP exerts its effects by means of complex and time-dependent signal transduction pathways. Moreover, it is increasingly recognized that loss of NPs effects is due to the phenomenon of "NPs resistance", namely the decrease or loss of NPs effects due to their repeated administration which, in turn, depend on Guanylate Cyclase Receptors (GCRs) down-

regulation, desensitization as well as the up-regulation of NPR-C, NPs scavenger receptor [28]. Furthermore, concentration dependence in the increase of ROS formation in BNP-treated cells at 3 h is not observed probably because BNP receptors become already fully saturated at $10^{-9}$ M, thus showing a maximal activity at this concentration.

Additionally, our results account for a BNP ability to give rise to an increase in arachidonic acid release by means of $cPLA_2$ involvement, in parallel with the hormone-induced ROS increase. This can be explained by the recent view that oxidative stress enhances $PLA_2$ activity in a number of systems, constituting a critical factor in inflammation [18]. Coherently, our experiments disclose a marked $LTB_4$ and $PGE_2$ release, accounting for BNP ability to both trigger $cPLA_2$-mediated AA release and its metabolites. Such a mechanism is conceivable, as MAFP, the selective $cPLA_2$ inhibitor, was able to totally inhibit these hormone effects. While $LTB_4$ accounts for a typical proinflammatory lipid, $PGE_2$ can act both as a proinflammatory and anti-inflammatory mediator depending on a context that is due, at least in part, to the array of prostaglandin receptors that exhibit opposing signalling pathways [19].

Moreover, it is well documented that $PGE_2$ can modulate cytokines production in several cell types [29,30], up-regulating the production of IL-10 by macrophages and potently inhibiting IL-12 and TNF-α release by dendritic cells (DCs) [31]. Interestingly, our results show that BNP is incapable of inducing proinflammatory cytokines release, namely TNF-α and IL-12, but it results in a marked IL-10 release, suggesting an anti-inflammatory cytokines profile induction. Finally, another point which deserves attention is represented by our data concerning BNP effect on macrophages migration. Our results, in fact, demonstrate that BNP is also able to increase the migratory activity of THP-1M$\phi$ after 24 h of stimulation. Since chemoattraction of macrophages into tissues is an essential step in the host response to infection [21], it would be facile to assume that our evidence point to a critical role for the hormone in mediating cell motility.

In summary, the ability of BNP to control three important aspects of macrophage activation such as the induction of ROS, $NO_2$ and $LTB_4$ production, may represent an evidence supporting a proinflammatory action of this cardiovascular hormone, whereas increases in BNP-induced $PGE_2$ and IL-10 levels may support an anti-inflammatory one. In conclusion, our results are the first to demonstrate a role for BNP as a direct determinant of macrophages activation rather than a paracrine mediator of tissue responses to inflammation-induced vascular stress. Although Dipeptidyl-peptidase IV can convert BNP (1–32) into its des-Ser-Pro form (3–32) [32], we believe that the effects observed are due to intact BNP because the peptidase IV is found soluble in plasma or expressed mainly on endothelial cells, epithelial cells and T-lymphocytes [33]; the only macrophage phenotype in which this enzyme has been found is alveolar macrophages [34], but yet no evidence on THP-1 macrophages has been reported. Furthermore, in a recent study, the effects of BNP (3–32) have been compared to those of BNP (1–32), showing reduced natriuretic, diuretic and vasoactive properties of BNP (3–32) [35]. This allows us to hypothesize a putative role for BNP as an important modulator of different processes involved in several inflammatory pathologies development, thus providing new perspectives in controlling those scenarios where increased BNP plasma levels have been reported.

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