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 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 (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 1-3x10^5 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; 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 μl 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 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 (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 μl) of concentrated

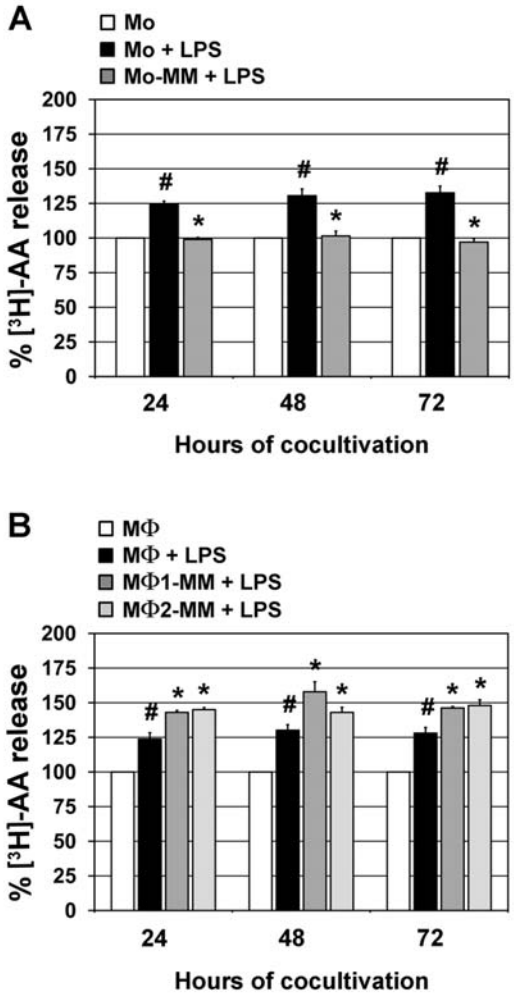

Figure 1. Effects of MM cells on the release of [^{3}H]-AA by THP-1 Mo and MΦ. (A) Percentile mean ± SD of [^{3}H]-AA release by unstimulated Mo, LPS-stimulated Mo and LPS-stimulated Mo-MM obtained by coculturing Mo with MM cells for 24, 48 and 72 h. (B) Percentile mean ± SD of [^{3}H]-AA release by 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 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 (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.

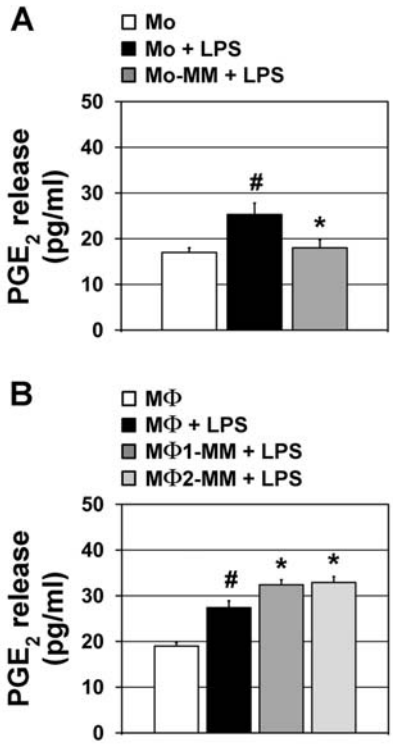

Figure 2. Effects of MM cells on PGE_2 release by THP-1 Mo and MΦ. (A) Mean ± SD values of PGE_2 release (pg/ml) by unstimulated Mo, LPS-stimulated Mo and LPS-stimulated Mo-MM obtained by coculturing Mo with MM cells for 24 h. (B) Mean ± SD values of PGE_2 release (pg/ml) by unstimulated MΦ, LPS-stimulated 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.

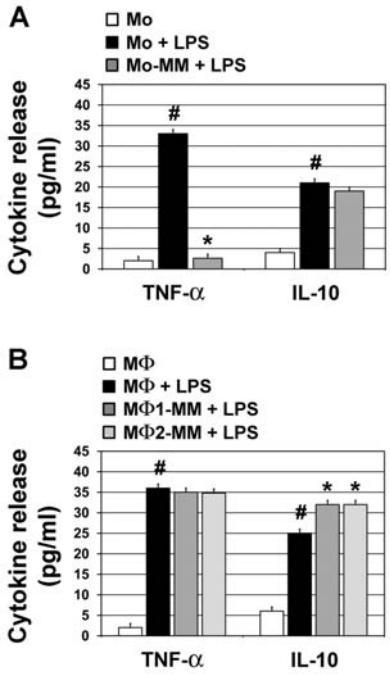

Figure 3. Effects of MM cells on TNF-α and IL-10 release. (A) Mean ± SD values of TNF-α and IL-10 release (pg/ml) by unstimulated Mo, LPS-stimulated Mo and LPS-stimulated Mo-MM obtained by coculturing Mo with MM cells for 24 h. (B) Mean ± SD values of TNF-α and IL-10 release (pg/ml) by unstimulated MΦ, 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,

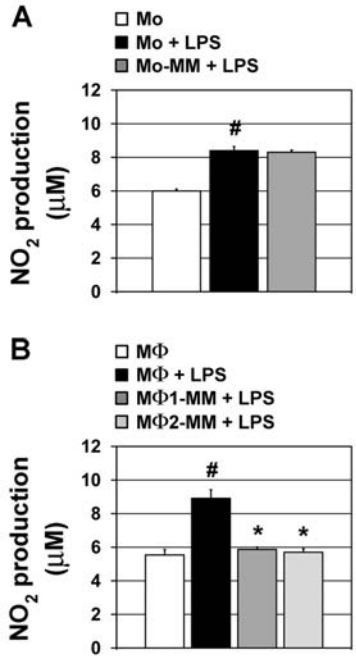

Figure 4. Effects of MM cells on NO_2 production. (A) Mean ± SD values of NO_2 (μM) produced by unstimulated Mo, LPS-stimulated Mo and LPS-stimulated Mo-MM obtained by coculturing Mo with MM cells for 24 h. (B) Mean ± SD values of NO_2 (μM) produced by unstimulated MΦ, LPS-stimulated MΦ, LPS-stimulated 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.

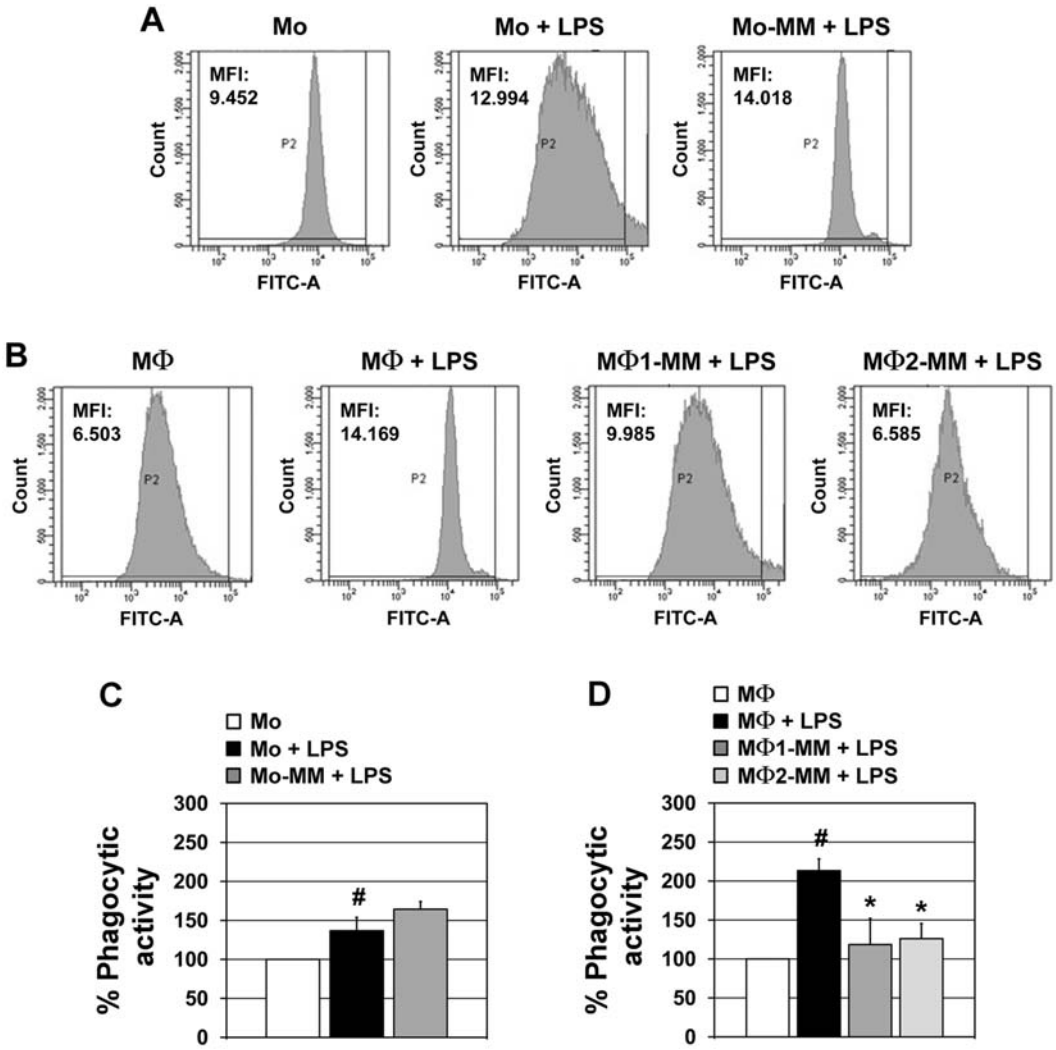

Figure 5. Effects of MM cells on Mo and MΦ phagocytic activity as assessed by flow cytometric analysis of FITC-dextran uptake. (A) A representative determination of FITC-dextran uptake by unstimulated Mo, LPS-stimulated Mo and LPS-stimulated Mo-MM obtained by coculturing Mo with MM cells for 24 h; the 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.

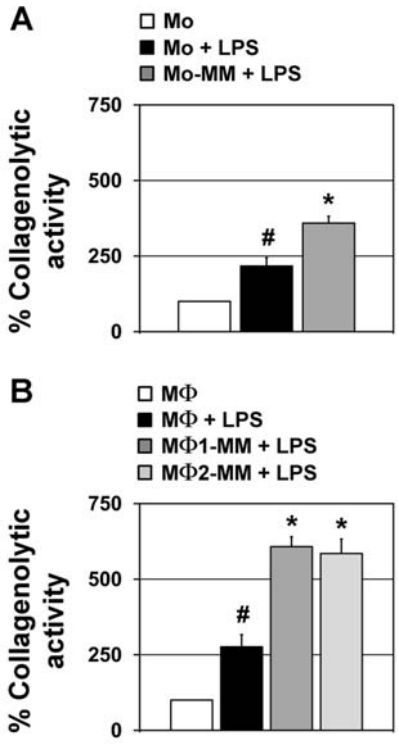

Figure 6. MM cells up-regulate the collagenolytic activity of THP-1 Mo and MΦ. (A) Percentile mean ± SD of the collagenolytic activity of unstimulated Mo, LPS-stimulated Mo and LPS-stimulated Mo-MM obtained by coculturing Mo with MM cells for 24 h. (B) Percentile mean ± SD of the collagenolytic activity 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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