Tumori, 97: 647-654, 2011

Influence of tumor cell culture supernatants on macrophage functional polarization: *in vitro* models of macrophage-tumor environment interaction

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ABSTRACT

Aims and background. Macrophages are heterogeneous cells with extensive functional plasticity; they can change their functional profiles repeatedly in response to environmental changes anywhere between their extreme phenotypical programs (labeled as M1 and M2 polarization, respectively). In terms of antitumoral immune response, M1 macrophages are considered to be beneficial, while M2 macrophages supposedly promote tumor progression. Tumor-associated macrophages (TAMs) represent a major leukocyte population present in many tumors. Although many studies indicate that TAMs elicit several M2-associated protumoral functions, including promotion of angiogenesis, matrix remodeling and suppression of adaptive immunity, their role regarding tumor progression is still controversial. The aim of the present study was to develop an appropriate *in vitro* model to study the effect of tumor-secreted soluble factors on the functional phenotype of macrophages.

Methods and study design. THP-1 human monocytic line cells and peripheral blood mononuclear cells from healthy volunteers were used for macrophage differentiation; primary tumor cell culture supernatants or tumor cell line supernatants were employed along with various cytokines, growth factors and other stimuli to design different model variants and to better mimic the *in vivo* tumor microenvironment.

Results. The cytokine secretion patterns of these macrophages suggest that primary tumor cell culture supernatants are able to switch the macrophage phenotype or to induce functional polarization of macrophages toward a mixed M1/M2 phenotype.

Conclusions. These data support the hypothesis that TAM behavior is modulated by the tumor microenvironment itself.

Key words: macrophages, M1/M2 polarization, tumor-associated macrophages, TAMs, tumor progression, cytokine pattern.

Conflict of interest: we declare we have no financial interests or other relationships which may lead to a conflict of interest.

Acknowledgments: This study was funded by the Romanian Agency for Scientific Research (contract grant number: 41-004).

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Received September 22, 2010;
accepted April 4, 2011.

Introduction

Macrophages are dynamic and heterogeneous cells. They display functional plasticity and can change their functional profiles repeatedly in response to environmental changes[1]. Mirroring the Th1/Th2 nomenclature, polarized macrophages are described as M1 or M2 cells. Macrophages can be induced to mount specific functional programs ranging anywhere between M1 and M2 polarization (considered as extreme phenotypical programs). M1 macrophages originate upon the encounter with cytokines such as granulocyte-macrophage colony stimulating factor (GM-CSF) and IFN-γ, or with microbial stimuli such as gram-negative bacteria lipopolysaccharide (LPS). They produce high levels of IL-12, IL-1, IL-6, IL-23, TNF-α, and CXCL10 and are characterized by activation of a polarized type I T-cell response, cytotoxic activity against microorganisms and neoplastic cells, expression of high levels of reactive oxygen intermediates, and antigen-presenting capability. On the other hand, monocytes differentiate into distinct

types of M2 cells after stimulation with macrophage colony stimulating factor (M-CSF), IL-4, IL-13, IL-10 or immunosuppressive agents (corticosteroids, vitamin D3, prostaglandins etc.). M2 cells secrete IL-10, CCL17, CCL22, CCL18, IL-1ra, and IL-1 decoy receptor and they have high scavenging ability, promote tissue repair and angiogenesis, and favor tumor progression[2-5].

Tumor-associated macrophages (TAMs) represent a major leukocyte population present in many tumors; however, their role regarding tumor progression is controversial[6-8]. Although most studies on different cancer types (including breast, prostate, bladder, cervical, ovarian, esophageal and kidney cancer) correlated high TAM density with poor survival, opposing data have been reported for other types of cancer such as gastric, lung and colorectal carcinomas[9-13]. Many studies indicate that TAMs elicit several M2-associated protumoral functions, including promotion of angiogenesis, matrix remodeling, and suppression of adaptive immunity[3,9,14,15]. The tumor environment is thought to "educate" TAMs towards an M2 phenotype, but the mechanisms underlying this phenomenon are not fully understood[16-18].

The aim of the present study was to develop an appropriate *in vitro* model to study the effect of soluble factors secreted by tumor cells on the functional phenotype of macrophages.

Material and methods

The design of the study was approved by the local ethics committee. In all cases where biological samples (tumor samples, blood) were needed from human subjects, written informed consent was obtained from patients as well as healthy volunteers and all the requirements concerning ethical aspects of using human biological samples in research experiments were thoroughly followed.

Isolation and culture of primary tumor cells from adenocarcinomas

Solid tumors were excised carefully and aseptically from pathologically proven colorectal and laryngeal cancer samples and transferred to the cell culture laboratory in AIM-V medium with streptomycin (50 µg/mL) and gentamycin (20 mg/mL) with a supplement of vancomycin (6 µg/mL) to prevent intestinal microflora contamination. The tumor tissues were transferred to a Petri dish and rinsed with fresh AIM-V medium. After dissecting necrotic areas, fatty tissues, blood clots, and connective tissues, tumor tissues were minced with sterile scalpels into small pieces of about 0.5-1 mm^3. To promote cell dissociation, the tissue pieces were homogenized by vigorous pippetting. Tumor cell aggregates were passed through a 70-µm-pore nylon cell strainer. After centrifugation, cells were resuspended in fresh complete medium and seeded onto 6-well plates. Cell cultures were maintained in a humidified incubator at 37 °C in an atmosphere of 5% CO_2 and 95% air.

Tumor cell culture supernatants

Primary tumor cell culture supernatants were collected depending on the growth characteristics. Usually, cancer cells grow *in vitro* as floating aggregates (spheroids), firmly/loosely adherent colonies, or as both adherent and floating subpopulations[19]. In the present study, since the primary colorectal cancer cells grew as spheroids, supernatants were collected 1 day after the spheroids became apparent under the optical microscope. Laryngeal cancer cell supernatants were collected 1 day after the cells reached 70-80% confluence (because the tumor cells had grown as both adherent and floating subpopulations). The SW403 culture supernatants were also collected 1 day after the cells reached 70-80% confluence.

Cell line cultures

The human monocytic cell line THP-1 and colon adenocarcinoma cell line SW403 were purchased from the Health Protection Agency Culture Collections. Both cell lines were cultured at 37 °C in 5% CO_2 in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 1 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin (complete culture medium).

Differentiation of THP-1 cells and polarization into M1 and M2 macrophage-like cells

THP-1 cells were differentiated into macrophages by treatment with 30 ng/mL phorbol-12-myristate-13 acetate (PMA) for 6 hours and then cultured for another 66 hours with either PMA and LPS (*E. coli* 055:B5 strain, 10 ng/mL)/IFN-γ (5 ng/mL) to generate M1-polarized cells, or with IL-4 (25 ng/mL) / IL-13 (25 ng/mL) to generate M2-polarized cells. Cells treated with PMA only (30 ng/mL) were used as controls. After 72 hours, the PMA-containing medium was removed and replaced by complete medium. Cells were maintained in culture with complete medium only for an additional 24 hours, and then supernatants were collected and stored at -20 °C until use. The protocol was adapted from a previously described method[20] (see *Discussion* for details).

Generation of human M1/M2 monocyte-derived macrophages (MDM)

Peripheral blood mononuclear cells (PBMCs) were isolated from the blood of healthy volunteers by standard density gradient centrifugation (30 minutes, 2000 rpm, 21 °C; Histopaque®-1077, Sigma-Aldrich) and washed 3 times with complete culture medium, each wash followed

by centrifugation for 10 minutes at 1500, 1200 and 1000 rpm, respectively, to reduce platelet contamination. PBMCs were resuspended in complete culture medium, counted, plated at 10×10^6/well (6-well flat bottom plates) and incubated for 2 hours (37 °C, 5% CO_2) to allow monocyte adherence. After 2 hours' incubation the nonadherent lymphocytes were removed and adherent monocytes were cultured for 7 days in complete culture medium supplemented with either 50 ng/mL recombinant human GM-CSF to generate M1 macrophages or 50 ng/mL recombinant human M-CSF to generate M2 macrophages. On day 4, 1.5 mL of medium was replaced with 1.5 mL fresh medium with cytokines. On day 7 macrophages were harvested, counted and seeded in duplicate at 2×10^5 cells/mL, 200 μL/well in 96-well flat-bottom culture plates and stimulated for 24 hours with LPS (1 μg/mL) and IFN-γ (20 ng/mL). Cells cultured without LPS and IFN-γ were used as controls. The supernatants were collected after 24 hours and stored at -20 °C until use. The protocol was adapted from a previously described method[21].

Culture of human monocytes in the presence of tumor cell culture supernatants

THP-1 cells were differentiated into macrophages by treatment with PMA (30 ng/mL) for 72 hours. Six hours after PMA stimulation, tumor cell culture supernatants (20% of tumor cell culture medium v/v) were added and cells were thus cultured for the remaining 66 hours. After 72 hours, the medium was removed and replaced by complete medium. Cells were maintained in culture with complete medium only for an additional 24 hours, and then supernatants were collected and stored at -20 °C until use. Human monocytes isolated as described above were cultured for 7 days with GM-CSF or M-CSF in the presence of tumor cell culture supernatants (20% of cell culture medium v/v). On day 4, 1.5 mL of medium was replaced with 1.5 mL fresh medium containing cytokines ± tumor cell culture supernatants. On day 7, macrophages were harvested, counted and seeded in duplicate at 2×10^5 cells/mL, 200 μL/well in 96-well flat-bottom culture plates and stimulated for 24 hours with LPS (1 μg/mL) and IFN-γ (20 ng/mL). Cells cultured without LPS and IFN-γ were used as controls. The supernatants were collected after 24 hours and stored at -20 °C until use.

Cytokine profile of the macrophages

To assess the M1/M2 polarization of THP-1 macrophages, the concentrations of TNF-α, IL-1ra and IL-6 were determined in culture supernatants. To assess the M1/M2 polarization of MDMs, the concentrations of TNF-α, IL-6, IL-10 and IL-12 (p70) were determined in culture supernatants. The cytokine levels were measured by ELISA (DuoSet kits from R&D Systems Inc., Minneapolis, MN, USA) according to the manufacturer's instructions.

Statistical analysis

Statistical analysis was performed using a paired Student's t-test. *P* values of less than 0.05 were considered significant.

Results

Functional polarization of human THP-1 cells into M1 and M2 macrophage-like cells and the influence of tumor cell culture supernatants

M1-polarized THP-1 macrophages were obtained by treatment of THP-1 cells with PMA for 72 hours (PMA only for 6 hours and then PMA + IFN-γ/LPS for the remaining 66 hours). M2-polarized THP-1 macrophages were obtained by treatment of THP-1 cells with PMA for 72 hours (PMA only for 6 hours and then PMA + IL-4/IL-13 for the remaining 66 hours). THP-1 cells treated with PMA only for 72 hours served as control cells. M1-polarized THP-1 macrophages secreted significantly higher levels of TNF-α, IL-6 and IL-1ra than did control cells ($P<0.001$ for TNF-α and $P<0.05$ for IL-6 and IL-1ra) (Figure 1A, B, C); M2-polarized THP-1 macrophages secreted significantly higher levels of IL-1ra only ($P<0.001$) (Figure 1C) while TNF-α levels were significantly lower ($P<0.05$) (Figure 1A) and IL-6 levels were comparable to control values (Figure 1B). These results delineate 2 opposite patterns, an M1-like pattern upon stimulation with LPS/IFN-γ with significantly higher values for TNF-α and IL-6 and significantly lower values for IL-1ra, and an M2-like pattern upon IL-4/IL-13 stimulation ($P<0.05$ in all cases).

To investigate the effects on THP-1 polarization, 3 different supernatants were used: 1 colon adenocarcinoma cell line SW403 supernatant (SW403) and 2 different primary colorectal cancer cell supernatants (S1, S2). When cultured with both primary tumor cell culture supernatants, THP-1 macrophages secreted significanly higher levels of TNF-α, IL-6 and IL-1ra than did control cells ($P<0.001$ for TNF-α and IL-1ra and $P<0.05$ for IL-6 for both supernatants) (Figure 1A, B, C). By contrast, THP-1 macrophages cultured with SW403 supernatant secreted higher levels of TNF-a only ($P<0.05$) (Figure 1A) while the levels of IL-6 and IL-1ra were comparable to control values (Figure 1B, C). Apparently, both tumor supernatants induced a peculiar pattern with TNF-α and IL-6 values similar to the M1-like pattern but with high values of IL-1ra, similar to the M2-like pattern ($P<0.05$ as compared to IL-4/IL-13 stimulation).

Functional polarization of human monocytes into M1 and M2 MDMs and the influence of tumor cell culture supernatants

The M1/M2 MDM model implied differentiation of human blood monocytes for 7 days with the lineage determining cytokines GM-CSF and M-CSF, respectively; cells

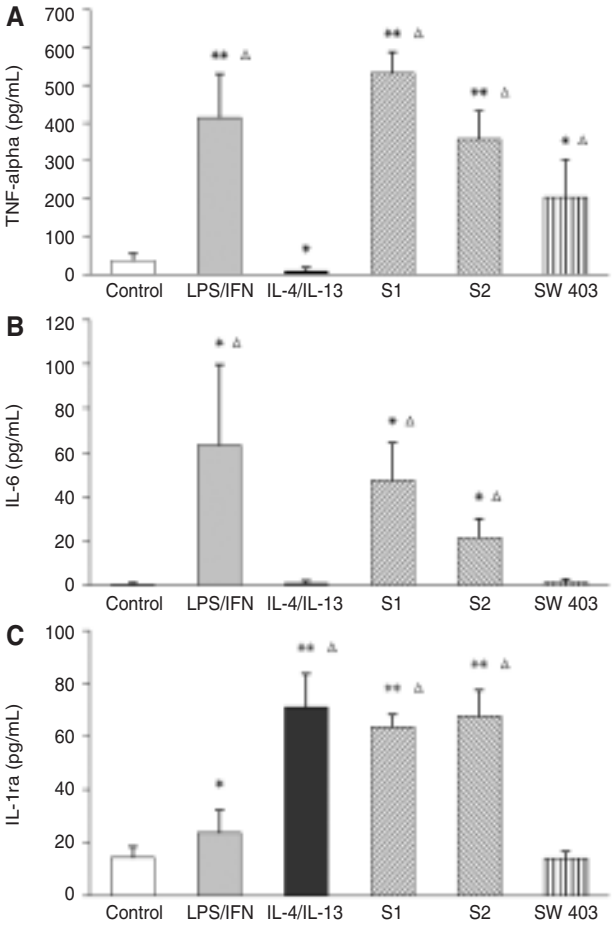

Figure 1 - Cytokine secretion by THP-1 macrophages. THP-1 cells were treated with: PMA only (30 ng/mL) for 72 hours ("control"), PMA + LPS/IFN-γ (PMA only for 6 hours and then PMA + IFN-γ/LPS for the remaining 66 hours, 30 ng/mL PMA, 10 ng/mL LPS and 5 ng/mL IFN-γ, "LPS/IFN"), PMA + IL-4/IL-13 (PMA only for 6 hours and then PMA + IL-4/IL-13 for the remaining 66 hours, 30 ng/mL PMA, 25 ng/mL IL-4 and 25 ng/mL IL-13, "IL-4/IL-13"), PMA + primary colorectal cancer cell supernatants obtained from 2 different cancer samples (PMA only for 6 hours and then PMA + supernatant for the remaining 66 hours, 30 ng/mL PMA, 20% of tumor cell culture supernatant v/v, "S1" and "S2", respectively) and PMA (PMA only for 6 hours and then PMA + a colon adenocarcinoma cell line [SW403] supernatant for the remaining 66 hours, 30 ng/mL PMA, 20% of cell culture supernatant v/v, "SW403"). The concentrations of TNF-α (A), IL-6 (B) and IL-1ra (C) were determined in THP-1 culture supernatants. Each bar represents the mean ± standard deviation of 3 independent experiments performed in duplicate. *$P \leq 0.05$; **$P \leq 0.001$ compared to control (Student's 2-tailed t-test); $^{\Delta}P \leq 0.05$ compared to "IL-4/IL-13" (Figure 1A and B); $^{\Delta}P \leq 0.05$ compared to "LPS/IFN" (Figure 1C).

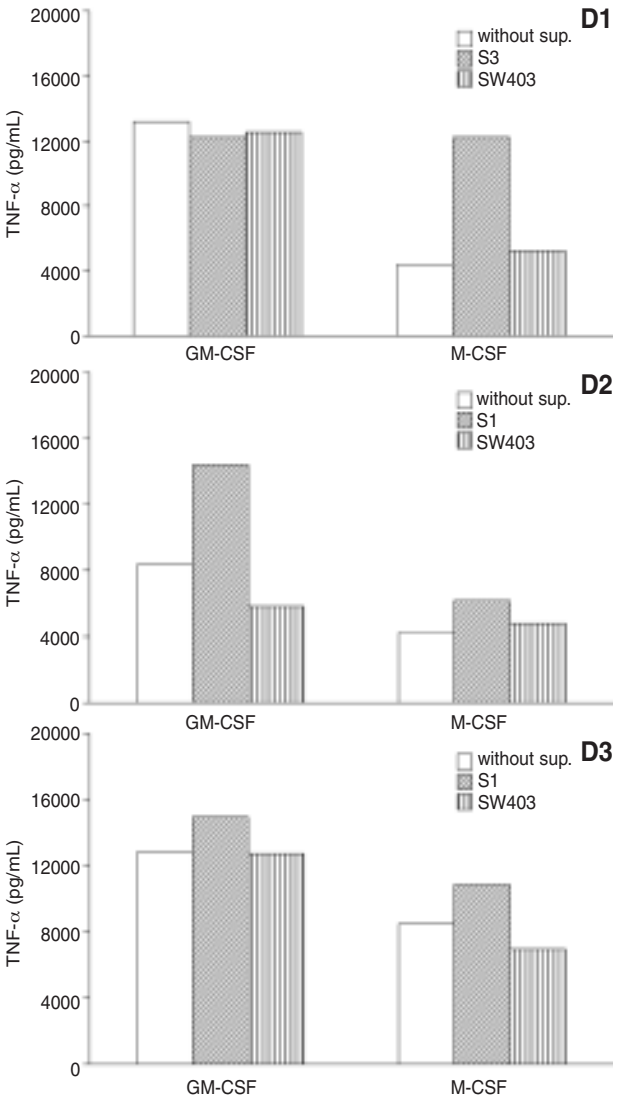

Figure 2 - TNF-α secretion by MDMs. Human monocytes isolated from the blood of 3 healthy volunteers (D1, D2, D3) were cultured for 7 days with 50 ng/mL GM-CSF ("GM-CSF") or 50 ng/mL M-CSF ("M-CSF") in the presence or absence of colon adenocarcinoma cell line SW403 supernatant ("SW403"), primary colorectal cancer cell supernatant ("S1") or primary laryngeal cancer cell supernatant ("S3"). Day 7 macrophages were stimulated for 24 hours with 1 μg/mL LPS + 20 ng/mL IFN-γ ("LPS/IFN"). After 24 hours, the TNF-α concentration was determined in MDM culture supernatants. Each bar represents the value obtained with cells from 1 healthy donor.

were stimulated with LPS + IFN-γ and TNF-α (Figure 2), IL-12p70 (Figure 3), IL-10 (Figure 4) and IL-6 levels were determined in culture supernatants. Cells differentiated with GM-CSF or M-CSF but without LPS/IFN-γ stimulation were used as controls (values not shown for clarity).

To investigate the effects on macrophage polarization, 3 supernatants were used: a colon adenocarcinoma cell line SW403 supernatant (SW403), a primary colorectal cancer cell supernatant (S1), and a primary laryngeal cancer cell supernatant (S3).

Cells cultured with GM-CSF + S1/S3 supernatants secreted lower levels of IL-12 than cells cultured with GM-CSF only. Also, cells cultured with M-CSF + S1/S3 supernatants secreted slightly lower levels of IL-10 than cells cultured with M-CSF only. Only cells cultured with GM-CSF + S1 (and not S3) supernatant secreted higher levels of TNF-α than cells cultured with GM-CSF only. Cells cultured with M-CSF + S1/S3 supernatants secreted higher levels of TNF-α than cells cultured with M-CSF only.

Cells cultured with SW403 supernatant in both conditions (GM-CSF or M-CSF) behaved similarly to S1/S3 cul-

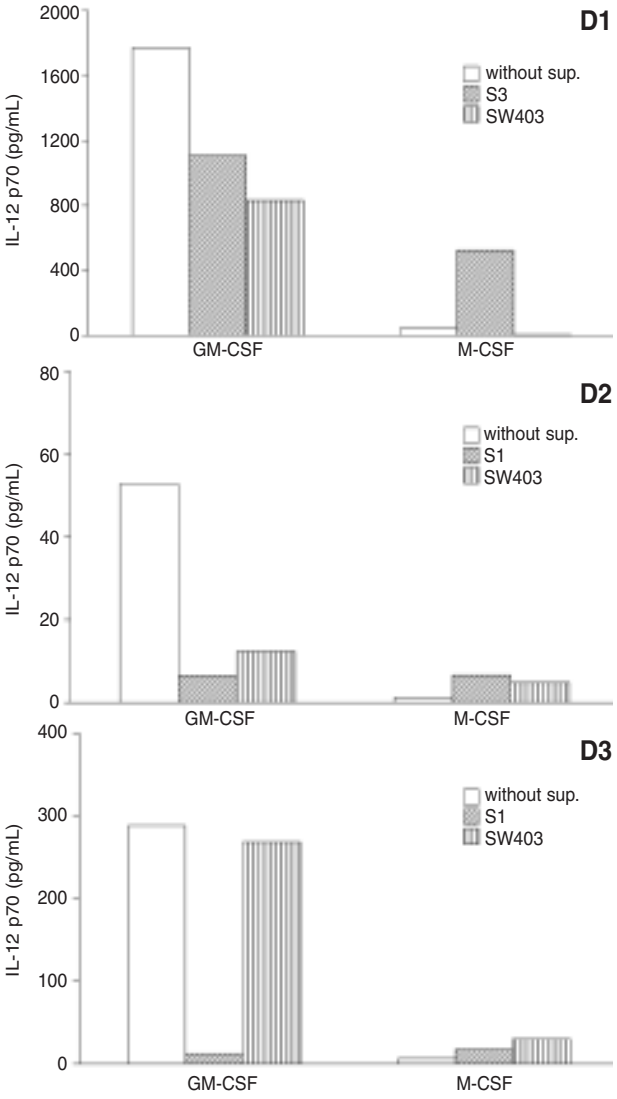

Figure 3 - IL-12 secretion by MDMs. Human monocytes isolated and cultured as described for TNF-α. After 24 hours' stimulation, the IL-12p70 concentration was determined in MDM culture supernatants. Each bar represents the value obtained with cells from 1 healthy donor.

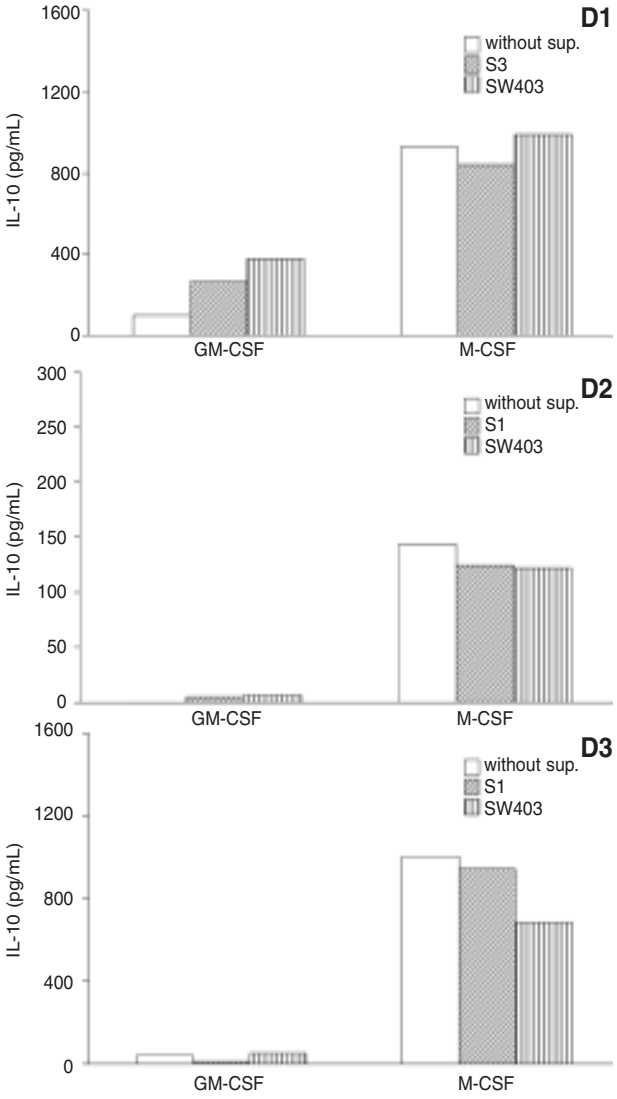

Figure 4 - IL-10 secretion by MDMs. Human monocytes isolated and cultured as described for TNF-α. After 24 hours' stimulation, the IL-10 concentration was determined in MDM culture supernatants. Each bar represents the value obtained with cells from 1 healthy donor.

tured cells in respect to IL-12 and IL-10. However, cells cultured with SW403 secreted comparable levels of TNF-α to cells cultured with GM-CSF and M-CSF only. There were no differences in IL-6 secretion by cells cultured with any of the supernatants (S1, S3 or SW403) in all differentiation conditions (data not shown).

Considering the results from a different perspective, GM-CSF induced a different cytokine pattern than M-CSF for all determined cytokines (higher levels of TNF-α and IL-12p70 and lower levels of IL-10 for GM-CSF as compared to M-CSF), with or without SW403 treatment. However, S3 treatment was associated with a change in these patterns.

MDM morphology after stimulation with LPS and IFN-γ differed according to the differentiation conditions and treatment with supernatants (Figure 5).

Furthermore, M-CSF only or in addition with SW403 supernatant induced a specific morphology, with most cells adopting an amoeboid morphology and few round cells (Figure 5A, B); S3 supernatant induced a shift toward a fibroblastoid morphology (mixed amoeboid and fibroblastoid cells) (Figure 5C). In contrast, GM-CSF only or in addition with SW403 supernatant induced a mixed morphology, with both round and fibroblastoid cells (Figure 5D, E), while with S3 supernatant most macrophages adopted a clear fibroblastoid morphology (Figure 5F).

Discussion

According to recent literature data, macrophages, which usually make up a significant part of the tumor-infiltrating immune cells, can considerably affect the course

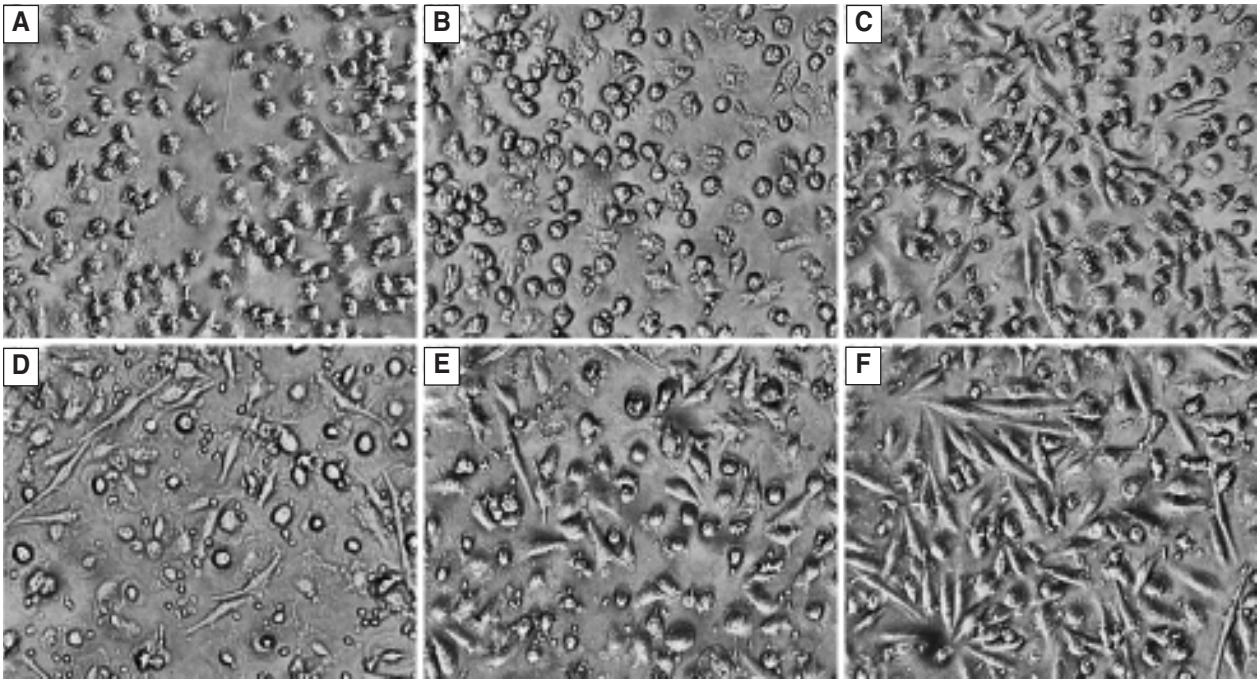

Figure 5 - Morphology of MDMs. Human monocytes were cultured for 7 days with 50 ng/mL M-CSF, either alone (A) or in the presence of colon adenocarcinoma cell line SW403 supernatant ("SW403") (B) or a primary laryngeal cancer cell supernatant ("S3") (C). The same protocol was followed for human monocytes cultured for 7 days with 50 ng/mL GM-CSF, either alone (D) or in the presence of colon adenocarcinoma cell line SW403 supernatant ("SW403") (E) or a primary laryngeal cancer cell supernatant ("S3") (F). In all cases, macrophages were stimulated for the last 24 hours with LPS (1 μg/mL) + IFN-γ (20 ng/mL). Original magnification ×400.

of cancer[4,7,22]. Due to the functional plasticity of macrophages, the phenotype of TAMs is strongly influenced by microenvironmental factors present within the developing tumor, which appear to promote their protumoral functions[9]. Several studies suggest that macrophages that infiltrate tumors acquire the properties of a polarized M2 phagocyte population (with the ability to release various factors such as platelet-derived growth factor, fibroblast growth factor-basic, epidermal growth factor, vascular endothelial growth factor, IL-10, transforming growth factor-β and several matrix metalloproteases); however, this might be an oversimplification[22,23].

The aim of the present study was to design an accessible and convenient *in vitro* model to study macrophage morphology and functions in relation to the tumor microenvironment. However, the "M1" and "M2" concepts are ill-defined and most probably experimental models can only reflect reality to a limited extent. Consequently, we employed 2 different models (although the model using donor monocytes was impaired by interindividual variations). Furthermore, the 2 models had different goals:

- using a THP-1-derived macrophage model we aimed to show that primary tumor cell culture supernatants are able to influence functional polarization of macrophages;
- using a donor monocyte-derived macrophage model we aimed to show that primary tumor cell culture supernatants are able to switch the macrophage phenotype.

Specifically, the first model comprised macrophage polarization and analysis of the effect of tumor-secreted soluble factors on macrophage phenotype. The human THP-1 cell line is widely used in monocyte/macrophage models and is a suitable alternative to PBMCs because of the high growth yield and reproducibility but also the lack of donor-related variability. In this study M1- or M2-polarized THP-1 macrophages were generated by differentiating THP-1 cells with PMA for 72 hours and polarizing them with either IFN-γ/LPS or IL-4/IL-13 (PMA only for 6 hours and then PMA + IFN-γ/LPS or IL-4/IL-13 for the remaining 66 hours, respectively). The PMA concentration was 6 times lower than with the method described by Tjiu *et al.*[20] to prevent gene expression upregulation by higher PMA concentrations, which might obscure gene expression induced by other stimuli as suggested by some authors[24]. Also, PMA treatment was extended from 24 to 72 hours because the 24-hour treatment rendered unstable cells that were easily detachable by mere washing. Furthermore, M1-polarized cells were generated using lower IFN-γ/LPS concentrations, because a gradual loss of viability was noticed with higher concentrations (data not shown). THP-1 cells were kept in a resting state for 24 hours to allow recovery after PMA treatment and to limit constitutive cytokine production (data not shown). THP-1-derived macrophages subsequently activated with LPS/IFN-γ secreted significantly higher levels of all tested cytokines compared to control values; the highest val-

ues were noticed for proinflammatory cytokines (TNF-α and IL-6), framing an M1-like pattern. By contrast, when stimulated with IL-4/IL-13, THP-1 macrophages secreted significantly higher levels of IL-1ra only (M2-like pattern), while TNF-α levels were significantly lower and IL-6 levels were comparable to control values.

When cultured in the presence of primary tumor cell culture supernatants, THP-1 macrophages expressed a distinct secretion pattern with both M1 and M2-like characteristics, showing high levels of the proinflammatory cytokines TNF-α and IL-6 but also of IL-1ra. As expected, there were differences between supernatants of primary colorectal cancer cells and the adenocarcinoma cell line SW403, the latter inducing lower levels of all tested cytokines compared to controls. As mentioned in a previous study, homogeneous lines are very different from primary cultures and do not reflect the *in vivo* tumor microenvironment[19].

The tumor microenvironment is characterized by a cytokine pattern which is likely to play a central role in the orientation and differentiation of recruited mononuclear phagocytes (and in particular of TAMs), thus contributing to direct the local immune system away from antitumor functions[9]. Proinflammatory cytokines such as TNF-α, IL-1β and IL-6 appear to be beneficial for the tumor by contributing to angiogenesis, immune suppression and cancer cell metastatic potential[5]. TNF-α can suppress the apoptosis of cancer cells and stimulate their proliferation through NF-κB activation. By increasing the vascular permeability, it can enhance the recruitment of leukocytes as well as intravasation and extravasation of cancer cells[25]. Also, cocultivation of tumor cells with macrophages leads to enhanced invasiveness of the malignant cells because of TNF-α-dependent matrix metalloprotease induction in the macrophages[6]. Other studies highlighted the role of tumor-secreted factors such as versican (secreted by metastatic carcinoma cell lines), which is responsible for secretion of high amounts of IL-6 and TNF-α by bone marrow-derived macrophages through activation of the Toll-like receptor family members TLR2 and TLR6[25]. Cocultures of macrophages and colon cancer cells in an *in vitro* model showed that tumor cells first stimulated macrophages to produce IL-6, which subsequently induced production of IL-10 (a cytokine which exhibits various immunosuppressive effects) by colon cancer cells[26]. Cytokines present in the tumor microenvironment such as IL-6 and IL-10, but also VEGF might contribute to TAM accumulation[27]. Apparently, TAMs derive from circulating monocytes that are recruited into tumors by chemotactic factors such as M-CSF and CCL2. These cells show a remarkable degree of plasticity during tumor development, with a switch in macrophage phenotypes occurring during the course of tumor progression[28]. Therefore, TAMs might display an M1 phenotype in sites of chronic inflammation associated with tumor development and an M2 phenotype in already established tumors. Furthermore, there is experimental evidence for TAMs expressing both M1- and M2-like characteristics in some forms of established tumors[28]. In line with these results, in our hands, THP-1-derived macrophages cultured with primary tumor cell supernatants expressed a mixed cytokine pattern with both M1- and M2-like characteristics. Thus, the proposed model proved its utility for studying the influence of tumor-secreted factors on inducing different functional phenotypes of macrophages.

In the second model, to better mimic the *in vivo* conditions, human PBMCs isolated from healthy volunteers were used and growth factors (GM-CSF and M-CSF) were used as differentiation agents. The cells were stimulated with LPS/IFN-γ in order to detect differences regarding the cytokine profile of the GM-CSF- and M-CSF-treated macrophages (undetectable in unstimulated cells). We showed that after 24 hours of stimulation, the macrophage phenotype could still be characterized as M1 (for GM-CSF) or M2 (for M-CSF) with regard to TNF-α, IL-12p70 and IL-10. In accordance with literature data[21,29], the determined cytokine profile of GM-CSF treated cells comprises high IL-12 and TNF-α and low IL-10 (M1-like profile), which is the exact opposite of the M2-like profile of M-CSF-treated cells. Treatment with S1 or S3 supernatant (supernatants from different cancer cell types than in the THP-1 model were used to rule out the bias induced by a particular cancer type) was associated with a change in these profiles for both GM-CSF and M-CSF conditions, suggesting that certain tumor-secreted factors might promote a phenotypic switch from M1- or M2-like to mixed M1/M2 profiles. These results are in accordance with those of other studies. For example, it was reported that human macrophages cocultured with ovarian carcinoma cell lines express high levels of both proinflammatory and antiinflammatory cytokines (TNF-α, IL-18, TGF-β1, and CCL22) together with M2 markers (mannose receptor, scavenger receptor)[30], suggesting the existence of a mixed macrophage phenotype in tumors[28]. In accordance with the cytokine profile, MDM morphology changed with the differentiation conditions: GM-CSF only induced a mixed morphology, with both round and fibroblastoid cells, while with GM-CSF + S3 most macrophages adopted a clear fibroblastoid morphology; M-CSF induced a different morphology with most cells adopting an amoeboid morphology and few round cells while M-CSF + S3 induced a shift toward a fibroblastoid morphology (mixed amoeboid and fibroblastoid cells). This second model might therefore be suitable for studying the influence of tumor-secreted factors on the MDM phenotype switch. However, these preliminary results have to be statistically validated by future experiments using monocytes from several donors.

In summary, both the THP-1-derived and donor monocyte-derived macrophage models, as uniquely depicted in this study, could be appropriate tools in TAM-related research. These *in vitro* models could enable a new and innovative approach to explore tumor-im-

mune system interaction. Furthermore, our results suggest that primary tumor cell culture supernatants are able to switch the macrophage phenotype or to induce functional polarization of macrophages toward a mixed M1/M2 phenotype. These data support the hypothesis that TAM behavior is modulated by the tumor microenvironment itself. Characterization of the phenotype of TAMs and identification of the nature of the factors secreted by tumor cells are therefore essential to better understand why high TAM densities are associated with either good or bad prognosis in different types of cancer and to identify new targets for therapeutic intervention.

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