Oncogene (2014) **33,** 2423–2431

www.nature.com/onc

ORIGINAL ARTICLE

Cancer-associated fibroblasts and M2-polarized macrophages synergize during prostate carcinoma progression

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Inflammation is now acknowledged as an hallmark of cancer. Cancer-associated fibroblasts (CAFs) force a malignant cross talk with cancer cells, culminating in their epithelial–mesenchymal transition and achievement of stemness traits. Herein, we demonstrate that stromal tumor-associated cells cooperate to favor malignancy of prostate carcinoma (PCa). Indeed, prostate CAFs are active factors of monocyte recruitment toward tumor cells, mainly acting through stromal-derived growth factor-1 delivery and promote their trans-differentiation toward the M2 macrophage phenotype. The relationship between M2 macrophages and CAFs is reciprocal, as M2 macrophages are able to affect mesenchymal–mesenchymal transition of fibroblasts, leading to their enhanced reactivity. On the other side, PCa cells themselves participate in this cross talk through secretion of monocyte chemotactic protein-1, facilitating monocyte recruitment and again macrophage differentiation and M2 polarization. Finally, this complex interplay among cancer cells, CAFs and M2 macrophages, cooperates in increasing tumor cell motility, ultimately fostering cancer cells escaping from primary tumor and metastatic spread, as well as in activation of endothelial cells and their bone marrow-derived precursors to drive *de novo* angiogenesis. In keeping with our data obtained *in vitro*, the analysis of patients affected by prostate cancers at different clinical stages revealed a clear increase in the M2/M1 ratio in correlation with clinical values. These data, coupled with the role of CAFs in carcinoma malignancy to elicit expression of stem-like traits, should focus great interest for innovative strategies aimed at the co-targeting of inflammatory cells and fibroblasts to improve therapeutic efficacy.

Oncogene (2014) **33,** 2423–2431; doi:10.1038/onc.2013.191; published online 3 June 2013

Keywords: prostate carcinoma progression; cancer-associated fibroblasts; cancer-associated macrophages; M1/M2 macrophage polarization; IL-6; SDF-1

INTRODUCTION

Cancer-associated fibroblasts (CAFs) are active players in tumor progression and metastatic spread, engaging with cancer cells a bidirectional interaction.[1,2] Cancer cells are involved in fibroblast activation through the secretion of several growth factors, including transforming growth factor-β (TGF-β), platelet-derived growth factor and interleukin (IL)-6. In turn, activated CAFs have a variety of effects on both cancer cells and the surrounding stroma, including alteration of extracellular matrix composition, *de novo* angiogenesis, as well as the commitment of cancer cells toward epithelial–mesenchymal transition (EMT) and their metabolic reprogramming toward a reverse Warburg phenotype.[3–5] EMT is an epigenetic transcriptional program by which epithelial cells gain mesenchymal features as reduced cell–cell contact and increased motility, thereby escaping the primary tumor and allowing dissemination of metastases at distance.[6,7] CAF-induced EMT has also been correlated with achievement of stem-like traits by cancer cells, and we recently involved a pro-inflammatory signature that exploits reactive oxygen species (ROS) to drive the migratory and aggressive phenotype of prostate carcinoma (PCa) cells. CAF-secreted metalloproteases elicit in carcinoma cells a Rac1b/cycloxygenase-2 (COX-2)-mediated release of ROS, which is mandatory for EMT, stemness and dissemination of metastatic cells.[5]

Cancer-associated macrophages (CAMs) represent the major inflammatory component of tumor stroma and have been involved in growth and progression of several tumors. Recent immunological studies have identified two distinct states of polarized macrophages activation: the 'classically' activated (M1) and the 'alternatively' activated (M2) macrophages. M1 macrophages have tumoricidal activity, produce high amounts of inflammatory cytokines, ROS and activate the immune response.[8,9] On the other hand, M2 macrophages promote tissue repair and angiogenesis, and favor tumor progression.[10–12] Many observations in pancreatic, colon and breast tumors indicate that CAMs show an M2 phenotype, and this preferential polarization is caused by absence of M1-directing signals such as interferon gamma (IFN-γ) or lipopolysaccharides (LPS), as well as by presence of M2-stimuli.[13–15] CAMs influence multiple steps in tumor development, including the growth, invasion and metastasis of tumor cells. Recently, Wu and colleagues[16] demonstrated that macrophages increase the migration and invasion of tumor cells by inducing EMT program through the nuclear factor-κB-mediated Snai1 stabilization. CAMs have also been reported to promote *de novo* angiogenesis in incipient *neoplasiae*. By these ways cancer cells can avoid nutrient/oxygen deprivation, activate the EMT transcriptional escaping program and find/recruit vessels to disseminate the tumor elsewhere. In a

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Received 25 March 2013; accepted 2 April 2013; published online 3 June 2013

mouse model of squamous skin carcinogenesis, CAFs exhibit a pro-inflammatory signature, leading them to secrete soluble factor and to recruit pro-angiogenic macrophages, thereby promoting tumor growth.[17]

In the present study, we aim to investigate the interplay between CAFs and CAMs in PCa progression. We identified a diabolic loop among CAFs, CAMs and PCa cells, in which interaction between stromal and cancer cells allows the setup of a pro-inflammatory microenvironment, due to the enrichment in reactive CAFs and M2-like macrophages. In turn, these stromal components actively contribute to enhance invasiveness of PCa cells, ultimately fostering cancer cells escaping from primary tumor and favouring metastatic spread.

RESULTS

Recruitment of human monocytes from PCa and its stroma

We have recently reported that PCa cells, in response to CAFs contact, undergo a clear EMT and acquire stem-like features associated with aggressiveness and metastatic spread.[4] Human prostate CAFs were isolated from the surgically explanted prostates of patients bearing PCa (Gleason $4+5$). As healthy counterparts, we used fibroblasts obtained from patients with benign prostate hyperplasia (human prostate fibroblasts, HPFs).[4] Aiming at investigating the effect of macrophages as a further component of tumor microenvironment, we isolated monocytes from normal human blood donors buffy coat and assayed the ability of PCa cells and CAFs to recruit them. Conditioned media (CM) from PCa cells or CAFs were used in a chemo-attraction assay, in which monocytes move toward CM in the bottom well of a Boyden chamber. The results show that both PCa cells and CAFs are able to efficiently recruit circulating monocytes (Figure 1a).

In order to identify the nature of signals originating by both PCa cells and CAFs to affect monocytes recruitment, we focused our attention on IL-6, monocyte chemotactic protein-1 (MCP-1) and stromal-derived growth factor-1 (SDF-1). Previous results indicated that MCP-1 and IL-6 are highly produced by PCa cells, whereas the activated stroma secretes large amount of SDF-1, metalloproteinases and IL-6.[18–21] In order to analyze the role of these factors in monocytes chemotaxis, we repeated the experiments, while inhibiting signals from IL-6, MCP-1 and SDF-1. To this aim, we used blocking antibodies toward IL-6 or MCP-1 in the bottom chamber of the chemotaxis assay, or blocking antibodies against CXCR4, the receptor for SDF-1, in the upper chamber of the same assay. We observed that the treatment with MCP-1-blocking antibodies is able to decrease the migration of monocytes induced by CM from PCa cells, as well as that CXCR4-blocking antibodies are active in CAFs-induced chemo-attraction. In contrast, we observed that treatment with IL-6-blocking antibodies is unable to affect the increase in migration induced by CM from PCa cells (Figure 1b).

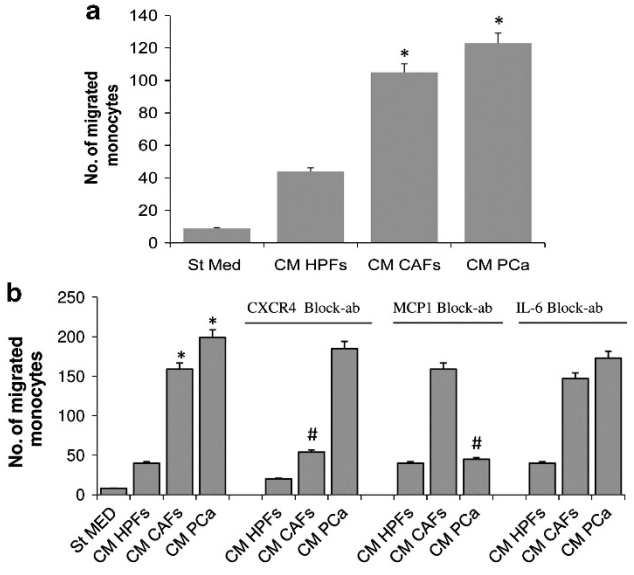


Figure 1. PCa and CAFs are able to recruit human monocytes. (**a**) Monocytes isolated from normal donor buffy coat were serum starved and then were allowed to migrate for 2 h toward CM from HPFs, CAFs or PCa. *$P<0.001$ versus St Med. (**b**) Monocytes were treated as in **a** but their migration was performed in the presence or absence of CXCR4- (10 μg/ml), IL-6- (200 μg/ml) or MCP-1-(130 μg/ml) blocking antibodies. *,$P<0,001$ versus St Med. $^{\#}P<0.001$ versus each correspondent control.

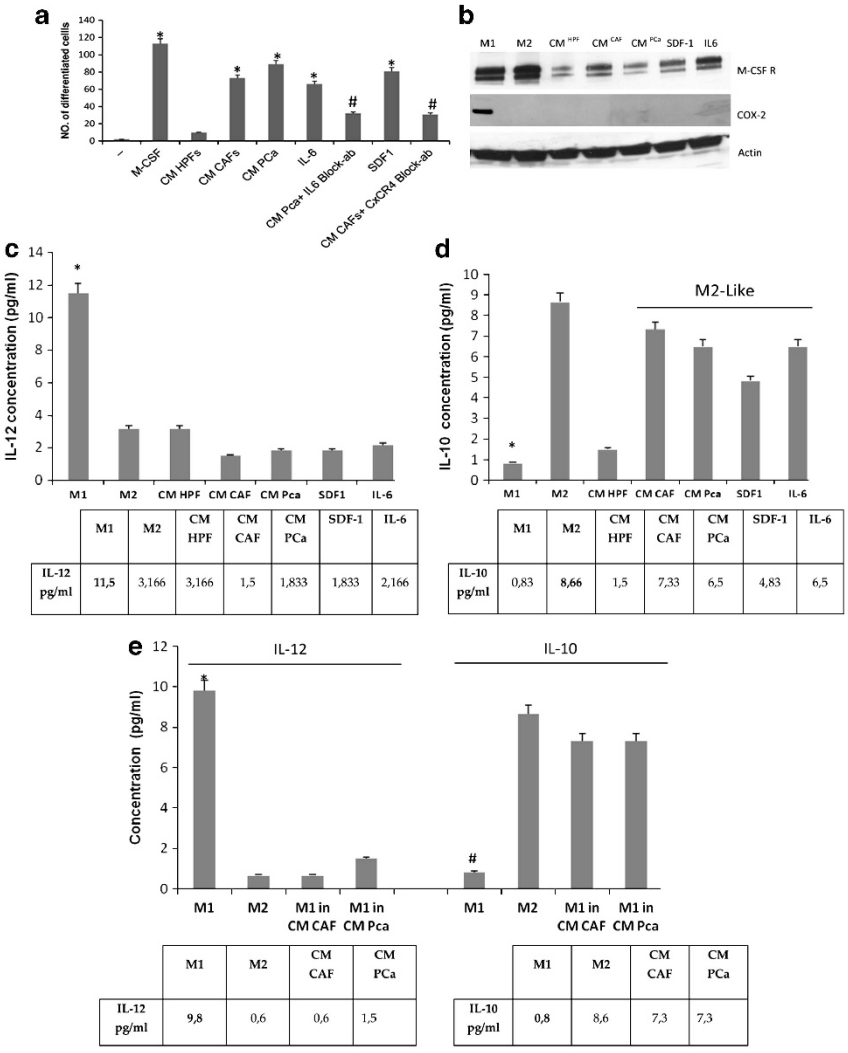
Differentiation of macrophages and M2 polarization from PCa and its stroma

In order to analyze if exposure to tumor microenvironment features can affect monocyte differentiation, we incubated human blood monocytes with CM from PCa cells or CAFs, using macrophage colony-stimulating factor (M-CSF) as control. The results show that both PCa cells and CAFs, but not HPFs, are able to induce evident morphological changes of monocytes, that became larger, with ruffling membrane typical of macrophages. Both IL-6 and SDF-1 are able to elicit similar effects (Figure 2a).

In order to analyze the phenotype of differentiated macrophages, we firstly assessed the expression of M-CSF receptor (M-CSFR) and COX-2. M-CSFR is highly expressed by macrophages cultured with M-CSF, as well as by macrophages treated with exogenous IL-6 or SDF-1, or incubated with CM from PCa cells or CAFs. In contrast, only macrophages stimulated with LPS in combination with IFN-γ (canonical M1 polarization) express high level of COX-2 (Figure 2b). As M1 macrophages are characterized by IL-12^{high} and IL-10^{low} production, whereas M2 macrophages are characterized by IL-12^{low} and IL-10^{high} production,[22] we evaluated the production of IL-10 or IL-12 in our experimental setting. The results reveal that macrophages differentiated by treating with CM from PCa or CM from CAFs, as well as macrophages treated with IL-6 or SDF-1 show an M2 phenotype (Figures 2b and c). Using specific-blocking antibodies, we confirm that IL-6 and SDF-1, but not MCP-1, released by stromal or PCa cells, are responsible for M2 polarization of macrophages (Figures 2a, c and d and Supplementary Figure 1). In addition, CM from PCa or from CAFs are both able to revert the canonical M1 polarization and to induce the *de novo* M2 polarization (Figure 2e), thereby confirming the strength of stromal or cancer-derived stimuli for macrophage polarization.

Macrophages differentiated by tumor or stromal cells affect PCa cells motility

In order to investigate the effects of macrophages on PCa cells motility, we treated cancer cells with CM from macrophages differentiated by contact with cancer cells, with HPFs or CAFs, or by treatment with IL-6 or SDF-1. Indeed, while IL-6 is not able to recruit monocytes to tumors, it is strongly produced by aggressive PCa cells[4] and is also able to mediate M2 macrophage polarization. SDF-1, produced by activated CAFs, is able to doubly recruit monocytes and differentiate them into the M2 phenotype. The analysis of PCa cells invasiveness, after treatment with CM from macrophages differentiated by the different treatments, reveals that macrophages are able to elicit a strong invasive spur in PCa cells, irrespectively by the stimuli they received to engage their differentiation or polarization (Figures 3a and b).



	M1	M2	CM HPF	CM CAF	CM PCa	SDF-1	IL-6
IL-12 pg/ml	11,5	3,166	3,166	1,5	1,833	1,833	2,166

	M1	M2	CM HPF	CM CAF	CM PCa	SDF-1	IL-6
IL-10 pg/ml	0,83	8,66	1,5	7,33	6,5	4,83	6,5

	M1	M2	CM CAF	CM PCa
IL-12 pg/ml	9,8	0,6	0,6	1,5

	M1	M2	CM CAF	CM PCa
IL-10 pg/ml	0,8	8,6	7,3	7,3

Figure 2. PCa and CAFs induce macrophage differentiation and M2 polarization. (**a**) Human monocytes were cultured for 7 days in CM from HPFs, CAFs or PCa cells in the presence or absence of CXCR4- (10 μg/ml) or IL-6-(200 μg/ml) blocking antibodies, or cultured with medium containing IL-6 (50 ng/ml) or SDF-1 (100 ng/ml). Monocytes were cultured for 7 days with M-CSF (50 ng/ml) as a positive control of differentiation. Differentiated macrophages were counted and a bar graph, representative of six randomly chosen fields, is shown. $^{*}P<0.001$ versus untreated. $^{\#}P<0.001$ versus each correspondent control. (**b**) M1 and M2 macrophages were obtained as reported in the Materials and methods section. In addition, monocytes were cultured for 7 days in CM from HPFs, CAFs or PCa cells and with medium containing IL-6 or SDF-1. Cells were lysed and the expression of M-CSFR, COX-2 and actin was evaluated by immunoblots. (**c**, **d**) Cells were treated as in **b** and the levels of IL-12 (**c**) or IL-10 (**d**) were measured by ELISA test. $^{*}P<0.001$ versus M2. (**e**) M1 and M2 macrophages were obtained as reported in the Materials and methods section. M1 macrophages were subsequently treated with CM from CAF or PCa cells for 48 h, and levels of IL-12 or IL-10 were measured by ELISA test. $^{*}P<0.001$ versus M2. $^{\#}P<0.001$ versus M2.

Macrophages are able to activate stromal fibroblasts to CAFs

We have observed that CAFs affect tumor malignancy by promoting EMT program, that is, the invasive and stem-like properties of cancer cells.[4] Hence, to complete our study on the cross talk among CAFs and macrophages, we focused our attention on the role of macrophages in activation of a reactive state of stromal fibroblasts. HPFs were either treated with CM from canonical M1 or M2 macrophages (after *in vitro* differentiation with IFN-γ/LPS or IL-4), as well as with CM from CAFs-induced M2-like macrophages, and α-SMA was assayed as a marker for fibroblast activation (Figure 4a). We compared fibroblast activation achieved through conventional TGF-β1 treatment and exposure to different CM. The results reveal that macrophages are able to elicit activation of HPFs to CAFs, suggesting a positive correlation between fibroblast activation and macrophages.

Finally, as a confirmation of the activated state of fibroblasts upon contact with macrophages, we evaluated the effect of corresponding CM on PCa invasiveness. The results show that fibroblasts activated by macrophages give a powerful motility spur to PCa, with a key role of IL-6 and SDF-1 in this mutual interplay (Figures 4b and c).

Pro-angiogenic effect of M2-like macrophages

In order to determine the involvement of macrophages obtained in our experimental conditions in *de novo* angiogenesis or vasculogenesis, we used either a mature endothelial cell population, that is, human umbilical vein endothelial cells (HUVEC) cells, or endothelial progenitor cells (EPCs), isolated from the blood of three human umbilical cords, immunophenotyped by flow

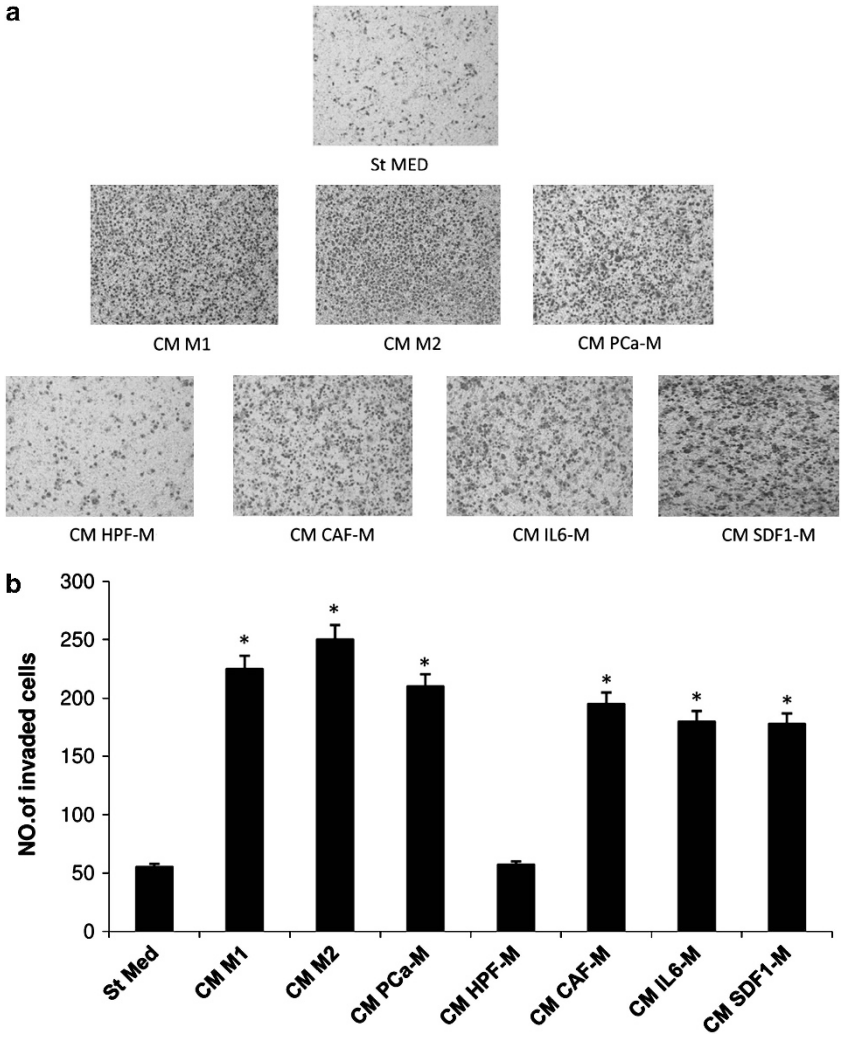


Figure 3. M2-like macrophages induce invasiveness in PCa cells. (**a**) Monocytes were differentiated for 7 days using CM from HPFs, CAFs or PCa cells, or differentiated with medium containing IL-6 (50 ng/ml) or SDF-1 (100 ng/ml). M1 and M2 macrophages were obtained as reported in the Materials and methods section. All the differentiated macrophages were then serum starved for 48 h to obtain the corresponding CM. PCa cells were incubated with CM from the above differentiated macrophages for 24 h, or serum starved as a control, and then were allowed to invade toward medium containing 10% serum as chemoattractant for additional 24 h. (**b**) Invading cells were counted and a bar graph, representative of six randomly chosen fields, is shown. *,$P < 0.001$ versus St Med.

cytometry.[23] Mature endothelial cells have been reported to drive *de novo* angiogenesis, whereas EPCs have been correlated with vasculogenesis, organized by tumor-recruited precursors.[24] We treated HUVEC or EPCs with CM from macrophages differentiated by contact with cancer cells, with HPFs or CAFs, or by treatment with IL-6 or SDF-1. For both cell populations, as sign of their commitment toward tube-like structure-forming cells, we assayed their ability to cross Matrigel barriers, as well as their ability to perform capillary morphogenesis. The results show that M2-like macrophages contribute to enhance invasiveness and tube-like structure formation in both EPCs and HUVECs cells, suggesting a key role of M2-like macrophages in driving vascularization of PCa (Figure 5). In keeping with other observations, M1 macrophages are less efficient in mediating this phenomenon.

M2 macrophages infiltration correlates with PCa aggressiveness

In order to evaluate the correlation between M2-polarized macrophages and PCa aggressiveness, we analyzed explants from 93 patients affected by clinically localized PCa with a minimum follow-up of 5 years. Clinical presentation, pathological findings and follow-up of the 93 patients included in our study are reported in (Supplementary Table 1). At the final anatomopathological evaluation, 35.5% of our study population presents an organ-confined disease, whereas in 64.5% of them, there is extracapsular extension. Massive infiltration of M1 macrophages occurs more frequently in organ-confined PCa (Gleason Score 6–7), whereas M2 macrophages are more represented in PCa with exracapsular extension (Gleason Score 7 to 8–10) (Figures 6a–d). At univariate analysis, M2 macrophages are statistically correlated to extracapsular extension ($P = 0.0079$) and it is also confirmed at multivariable Cox proportional hazard model ($P = 0.03$, relative risk 0.295, 95% confidence interval 0.09-0.89). At the Kaplan–Meier biochemical recurrence (BCR)-free survival analysis, when we stratify our study population for M1 and M2 macrophages occurrence, we observe that patients with prevalence of M2-polarized macrophages show a trend toward worst BCR-free survival rates at 36 and 60 months compared with patients with M1 prevalence (78.2 versus 94.1 and 71.0 versus 77.4, respectively)

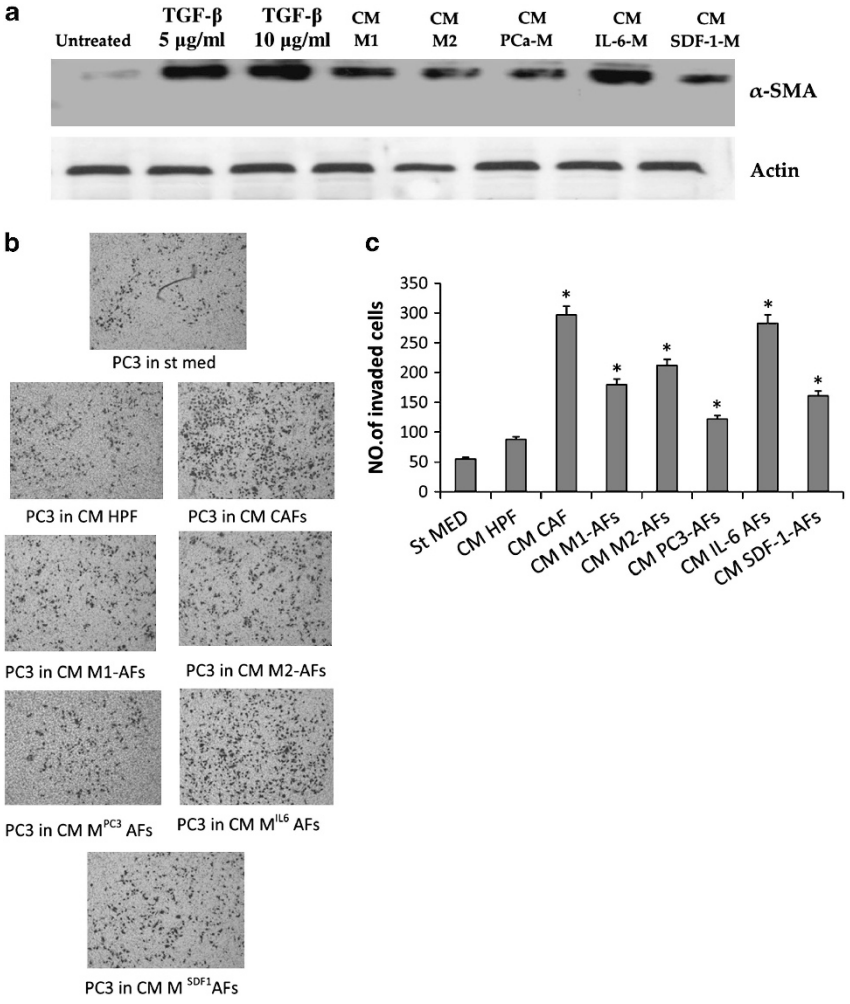


Figure 4. CAMs synergize with CAFs to increase PC3 motility. (**a**) Monocytes were differentiated for 7 days using CM from PCa cells, or with medium containing IL-6 (50 ng/ml) or SDF-1 (100 ng/ml). M1 and M2 macrophages were obtained as reported in the Materials and methods section. All the differentiated macrophages were then serum starved for 48 h to obtain the corresponding CM. Subconfluent HPFs were treated for 24 h with CM from the above differentiated macrophages or with 10 ng/ml TGF-β1 as a positive control, and serum starved for additional 24 h. Fibroblasts were lysed and immunoblots for α-SMA and actin were performed. (**b**) PCa cells were incubated for 24 h with CM from HPF treated as in **a** and then allowed to invade for additional 24 h toward medium containing 10% serum as chemoattractant. (**c**) Invading cells were counted, and a bar graph, representative of six randomly chosen fields, is shown. $*P < 0.001$ versus St Med.

(Figure 6e). Moreover, when we analyze survival curves for the category of patients with only extracapsular extension, those patients with M2 macrophages prevalence confirm to have a worse prognosis. Unfortunately, stratification for M1 and M2 macrophages do not allow us to establish a significant correlation with prognosis. Finally, in order to understand the role of M1-polarized macrophages in patients displaying high levels of M2 macrophages, we further analyze BRC-free survival rates of these individuals (M1high/M2high). The results indicate that these patients diverge by the M1low/M2high subgroup (Supplementary Figure 2), thereby suggesting a protective role of M1-polarized macrophages.

DISCUSSION

CAFs have been acknowledged as active factors in tumor progression of several neoplasiae. They have been involved in (i) eliciting EMT in cancer cells, thereby granting the achievement of stem-like traits,[4,25] (ii) the resistance to *anoikis* enhancing survival in bloodstream and finally culminating in successful metastatization,[26] (iii) favoring chemoresistance of cancer cells,[27,28] and (iv) exerting a trophic effect to tumor cells undergoing Warburg metabolism and supplying energy-rich metabolites to cancer cells.[3,29] Recently Erez and colleagues,[17,30] in a mouse model of squamous skin carcinogenesis, reported that CAFs mediated tumor-enhancing inflammation, promoting recruitment of macrophages, neovascularization and tumor growth in an nuclear factor-κB-dependent manner. Hence, CAFs are emerging as novel key factors in orchestrating tumor-promoting inflammation. Now, we involve them in the recruitment of monocytes toward tumor cells and in their M2 polarization. The M2 phenotype of macrophages, expressing a wide array of anti-inflammatory molecules, has been involved in providing an immunosuppressive microenvironment for tumor growth.[31,32]

The identification of molecules driving macrophage plasticity due to cancer microenvironment provides a basis for macrophage-focused diagnostic and therapeutic strategies.[33] Historically, Toll-like receptors and IL-4/IL-13 are the acknowledged signals regulating M2 polarization of macrophages, although recently some other subtypes of the M2 class have been described.[32] Prostate CAFs engage monocyte recruitment and their M2 polarization are mainly through SDF-1, a stromal factor absolutely mandatory for the cross talk among

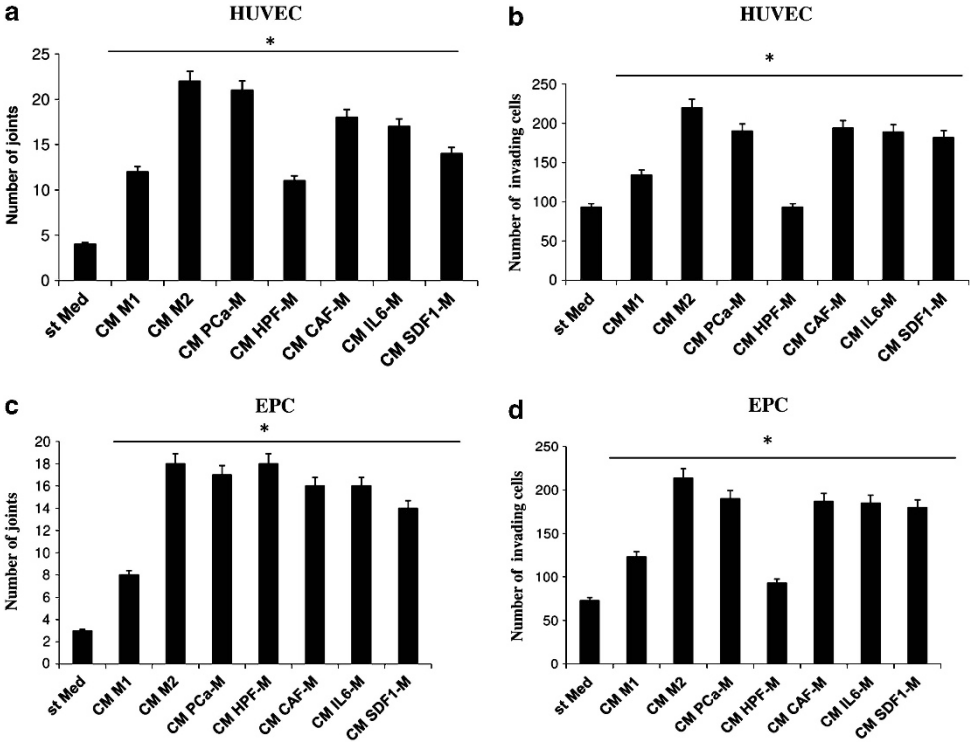


Figure 5. Pro-angiogenic effect of M2-like macrophages. (**a**, **b**) Monocytes were differentiated for 7 days using CM from HPFs, CAFs or PCa cells, or differentiated with medium containing IL-6 (50 ng/ml) or SDF-1 (100 ng/ml). M1 and M2 macrophages were obtained as reported in the Materials and methods section. All the differentiated macrophages were then serum starved for 48 h to obtain the corresponding CM. HUVEC cells were treated with CM from the above differentiated macrophages and *in vitro* angiogenesis was measured by capillary morphogenesis (quantified by measuring the number of joints as shown in **a** and by Matrigel invasion (quantified by counting the number of invading cells, as shown in **b**. $^*P<0.001$ versus St Med. (**c**, **d**) EPC cells were treated as in **a** and **b** respectively. $^*P<0.001$ versus St Med. Results are representative of three experiments with similar results.

CAFs and other cells of the tumor microenvironment, like bone marrow-derived endothelial precursors or mesenchymal stem cells.[20,21] Other soluble factors such as MCP-1 and CXCL14 have been reported to drive myeloid cell recruitment to the tumor site,[34] but their role in macrophage polarization has not been investigated. Our findings, adding *de facto* a further role to SDF-1 in monocyte attraction, confirm the key relevance of the axis SDF-1/CXCR4 for malignancy of several cancers,[35] including PCa, and acknowledge anti-SDF-1/CXCR4 pharmacological targeting as powerful antimetastatic tools. Of note, the known macrophage chemoattractant MCP-1, reportedly able to induce infiltration of blood monocytes in CAFs spheroids and to recruit monocytes into mammary tumors,[36–38] in our model is mainly produced by PCa cells and not by CAFs. Moreover, while MCP-1 is able to induce monocyte attraction, it is mostly unable to induce M2-macrophage polarization, likely leaving to SDF-1 this mandatory role. Alongside, IL-6, mainly produced by PCa cells in strict correlation with their aggressiveness,[4,39] as well as by CAFs, are able to both recruit monocytes and polarize them toward the M2 phenotype.

The relationships among cancer cells and their stromal cells are in most cases biunivocal. For CAFs, the biunivocal interplay has been divided in an 'afferent' pathway, driven by cancer-delivered factors affecting CAF reactivity and their activation, and the 'efferent' pathway, driven by activated CAFs secreting soluble factors, which in turn affect cancer cells aggressiveness, EMT and stemness.[1,4,40] A similar reciprocal relationship can be proposed for both CAFs and PCa cells with M2-polarized macrophages. Indeed, we found that M2 macrophages are able to activate healthy HPFs through mesenchymal–mesenchymal transition, and to convert them into a state similar to myofibroblasts or CAFs, able to promote PCa cell motility. This ability to activate HPFs to CAFs is shared by M2 macrophages polarized by contact with *ex vivo* CAFs, with cancer cells, as well as by IL-4-treated macrophages. Of note, the reciprocal interplay leads to enhanced invasiveness of PCa cells, suggesting a general cooperation within stromal, cancer cells and M2 macrophages to increase the malignancy of PCa cells. This cooperation embraces also endothelial cells or their bone marrow-derived precursors, which are pressed to organize into vessels.[41] Our *in vivo* data, obtained on a cohort of 93 patients with cT2b-c PCa treated with radical prostatectomy, strongly support this idea. Indeed, M1-polarized macrophages are prevalent in patients affected by organ-confined diseases, whereas M2-polarized macrophages resulted more represented in PCa with extracapsular extension. Univariate and multivariate analysis for M1/M2 occurrence confirms to be statistically correlated to ECE ($P=0.03$), whereas it fails to confirm their values as independent predictors of BCR. Above all, the Kaplan–Meier survival analysis confirms a worst BCR-free survival rate for patients with prevalence of M2-polarized macrophages.

We also found that M1-polarized macrophages, the so called classically activated macrophages, reportedly exerting antitumor effects, are able to increase the motility of PCa cells and to activate HPFs to CAFs, although to a lesser extent with respect to their M2-polarized companions. Of note, neither the contact with CAFs, nor with PCa cells, is able to elicit M1 polarization of macrophages, suggesting a marginal role of M1 macrophages in the effects exerted by tumor microenvironment. Hence, the presence of M1-polarized macrophages in aggressive tumors should be considered occasional, although we could not exclude that they

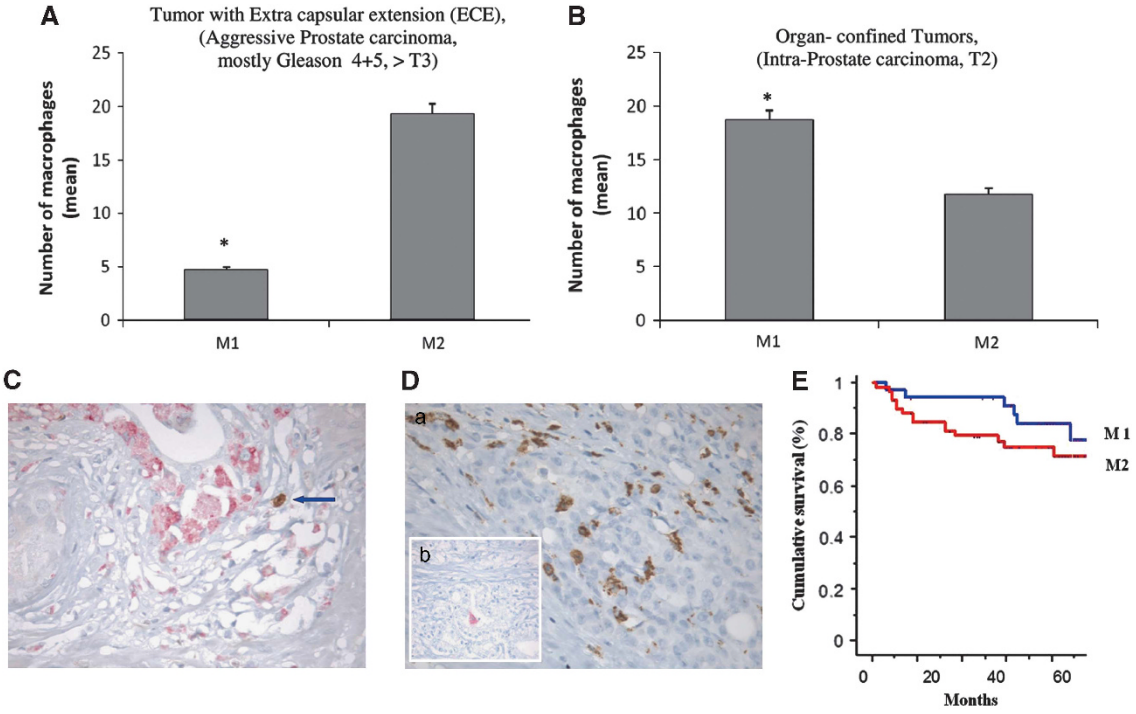


Figure 6. M2 macrophages infiltration correlates with PCa aggressiveness. (**A**, **B**) Prostatectomy specimens obtained from more than 100 patients were fixed in 10% formalin before being processed in paraffin. Hematoxylin and eosin-stained sections from each histological specimen were evaluated to obtain the histological diagnosis of prostatic adenocarcinoma according to the World Health Organization 2004 classification, the definition of the Gleason Grade, the perineural invasion and the evaluation of surgical margins. The tissue sections were stained with CD68 and CD163 antibodies. Macrophages were quantified by counting the M1 and M2 phenotypes in three hot spots. Finally, the mean of both the number of M1 and M2 macrophages in these three hot spots was obtained. *$P<0.001$ versus M2. (**C**) Presence of numerous M1 cells, characterized by a red and granular cytoplasm with chromogen fast red for CD68, and a single M2 cell (arrow) showing a brown cytoplasm with chromogen DAB for CD163. $\times$ 40 lens. (**D**) Area with a highest density of M2 macrophages in a Gleason Score 8 (4 + 4) prostatic adenocarcinoma: large number of M2 cells showing a brown and granular cytoplasm with chromogen DAB for CD163 (a, $\times$ 40 lens). The single M1 cell shows a red cytoplasm with chromogen fast red for CD68 in a different area of the same tumour (b, 40X lens). (**E**) BCR-free survival curves at the Kaplan–Meier analysis of our 93 patient study population stratified for M1 and M2 macrophages prevalence are represented in the graph. The blue line represents BCR-free survival curve of patients with M1 macrophage prevalence, whereas the red line represents those with M2 macrophage prevalence. BCR-free survival rates at 36 and 60 months were 94.1 versus 78.2 and 77.4 versus 71.0, respectively).

can enhance the motility of PCa cells as well, as our results *in vitro* indicate. Macrophages polarize to M1 phenotype in presence of infective agents, release of IFN-γ or stimulation of Toll-like receptors,[31,32,42] events that occurs only rarely within the tumor microenvironment. Conversely, M2 polarization occurring upon contact with CAFs, the main component of tumor stroma, or with cancer cells is highly feasible. Our further analysis of the subgroup of M2high patients, also showing high prevalence of M1-polarized macrophages (M2high/M1high), suggests that the presence of M1 macrophages is somewhat protective and, far to induce a worsening of prognosis, is correlated with organ-confined diseases and low markers of BCR.

Hence, in this complex web of relationships among different populations within tumor microenvironment, activation of CAFs is likely the 'primum movens' of these cross talks, and M2-polarized macrophages are master enhancers of stromal reactivity. Hence, if CAFs are activated in tumor stromal areas, this will lead to shift recruited macrophages toward the M2 phenotype, but in case of occasional presence of M1 macrophages a further activation of stromal CAFs is also possible, thereby further fostering monocyte attraction and their M2 polarization. Our findings on the ability of CAFs and PCa cells CM to induce a shift of M1-polarized macrophages toward the M2 phenotype, further support this idea.

Recently, tumor-associated macrophages have also been reported to interact with cancer stem cells.[43] This finding is in line with the key role exerted by CAFs in instructing macrophages during tumor progression to polarize toward the M2 phenotype. Indeed, CAFs contact with cancer cells is mandatory to elicit expression of stem-like markers in cancer cells, to sustain their ability to self-renew and to grow anchorage-independent spheroids.[4,5] Hence, the ability of tumor-associated macrophages to interact with cancer stem cells is likely the conclusion of the circuitry involving CAFs, M2 macrophages and cancer stem cells.

In conclusion, our data suggest the establishment of a fascinating loop among CAFs, M2-polarized macrophages and PCa, in which the intersected interaction between stromal and cancer cells allows for the setup of a pro-inflammatory micro-environment, due to the enrichment in reactive CAFs and M2-like macrophages. In turn, these stromal components actively contribute to enhance invasiveness of PCa cells, ultimately fostering cancer cells escaping from primary tumor and metastatic spread.

MATERIALS AND METHODS

Materials

Unless specified, all reagents were obtained from Sigma (Milano, Italy). Santa Cruz Biotechnology (Heidelberg, Germany) antibodies were as follows: COX-2, M-CSF R, β-Actin. Antibodies anti HIF-1α, CXCR4-blocking antibodies and anti-human MCP-1 were obtained from BD Transduction Laboratories (Milano, Italy); antibodies anti α-smooth muscle actin (α-SMA) were from Sigma. TGF-β1, IL-6, SDF-1, M-CSF and IL-4 were obtained from Peprotech (Hamburg, Deutschland, Germany). PVDF was obtained from

Millipore (Milano, Italy) and Matrigel was obtained from BD Biosciences (Milano, Italy).

Cell cultures

Human PCa cells (PC3) and HUVEC were obtained from the European Collection of Cell Cultures, were authenticated by PCR/short tandem repeat analysis (European Collection of Cell Cultures, Salisbury, UK) and was used within 6 months of resuscitation of original cultures.

PCa were cultured in DMEM containing 10% FBS, whereas endothelial cell were cultured on 1% gelatin- coated dishes in EGM-2 medium (Lonza, Basel, Switzerland). Endothelial progenitor cells (EPCs) have been isolated from human umbilical cord blood as previously described.[44,45] HPFs and CAFs were isolated from surgical explantations from patients affected by benign prostatic hyperplasia (HPFs) or from cancer regions of patients bearing PCa (Gleason $4+5$) (CAFs).[4]

Macrophages differentiation

Human monocytes were obtained from normal donor buffy coat by gradient centrifugation using Ficoll (Histopaque-1077). Non-adherent cells were removed and purified monocytes were incubated for 7 days in RPMI 1640 supplemented with 10% FBS and 50 ng/ml M-CSF to obtain macrophages. M0 cells were obtained by treating with serum-free medium for 48 h. M1 macrophages were polarized by stimulating overnight with LPS (100 ng/ml; Peprotech) and IFNγ (100 ng/ml; Peprotech). M2 macrophages were polarized by stimulating overnight with IL-4 (20 ng/ml; Peprotech). M2-like macrophages were obtain by culturing monocytes for 7 days in RPMI 1640 10% FBS in presence of IL-6 (50 ng/ml) or SDF-1 (100 ng/ml) or with 50% of CM from PCa or CAFs.

Preparation of CM

CM were obtained from untreated HPFs, CAFs, macrophages or PCa. Cells were grown to sub-confluence, then serum starved and incubated for 48 h before collection of the CM. CM were harvested, clarified by centrifugation and used freshly.

Western blot analysis

Fibroblasts, PCa cells or macrophages derived from our experimental conditions were lysed for 20 min on ice in 500 μl of RIPA lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 2 mM EGTA, 1 mM sodium orthovanadate, 1 mM phenylmethanesulphonyl-fluoride, 10 mg/ml aprotinin and 10 mg/ml leupeptin). Twenty micrograms of total proteins were loaded on SDS–PAGE, separated and transferred onto nitrocellulose. The immunoblots were incubated in 3% bovine serum albumin, 10 mmol/l Tris-HCl (pH 7.5), 1 mmol/l EDTA and 0.1% Tween 20 for 1 h at room temperature, probed first with specific antibodies and then with appropriate secondary antibodies.

In vitro Boyden invasion assay

PCa cell invasion was assayed with the Transwell system of Costar equipped with 8-μm-pore size and monocytes migration was evaluated with 5-μm-pore size polyvinylpyrrolidone-free polycarbonate filters. Migration or invasiveness assays are distinguished by the absence (migration assay) or the presence (invasiveness assay) of a three-dimensional barrier of Matrigel. A measure of 50 μg/cm^2 of reconstituted Matrigel (BD Biosciences) was added to the top chamber, allowed to solidify for 1 h at 37 °C and air dried for 16 h. The Matrigel barrier was rehydrated with 100 μl of DMEM for 2 h at 37 °C prior to use. Cells were loaded into the upper compartment (5×10^4 cells in 200 μl) in serum-deprived growth medium. The Matrigel invasion chambers were placed into 24-well culture dishes containing 500 μl of the different CM from monocytes as a chemoattractant. After 24 h of incubation at 37 °C, non-invading cells and the Matrigel layer were mechanically removed using cotton swabs, and the microporous membrane containing the invaded cells was fixed in 96% methanol and stained with Diff-Quick staining solutions. Chemotaxis was evaluated by counting the cells that migrated to the lower surfaces of the polycarbonate filters.

ELISA

Cytokine production in macrophages supernatants were measured by commercially available ELISA Kits (IL-10 and IL-12) according to the manufacturer's instructions (Invitrogen, Monza, Italy, KHC0104/ KHC0104).

Matrigel angiogenesis *in vitro*

All the experiments were performed using growth factor-reduced Matrigel at a concentration of 1 mg/ml. A measure of 50 μl of Matrigel were added to each well of a 96-well plate and then placed in a humidified incubator at 37 °C for 30 min. HUVECs and EPCs (2×10^4 cells/well) were added to the Matrigel-coated plates in a final volume of 200 μl. The effects on the morphogenesis of endothelial cells were recorded after 6 h with an inverted microscope equipped with CCD optics and a digital analysis system. Results were quantified by measuring the joint numbers in the field.

Patients characteristics

Clinical and pathological data were prospectively gathered from 93 patients treated with radical prostatectomy for clinically localized PCa January 2000 to December 2011. The follow-up schedule included serum PSA assay every 3 months for the first year, then every 6 months for the following 2 years and yearly thereafter. BCR was defined as evidence of PSA >0.2 ng/ml on two consecutive measurements. The BCR-free survival rate was estimated by the Kaplan–Meier method to establish the correlation between macrophages phenotype and prognosis. Statistical significance was verified by the log-rank test. Correlation between macrophages phenotype and other clinicopathological variables was investigated using the χ^2-test.

Tissue specimens and immunohistochemistry

The prostatectomy specimens were fixed in 10% formalin before being processed in paraffin. Hematoxylin and eosin-stained sections from each tissue block were evaluated to obtain the diagnosis of prostatic adenocarcinoma and the patological T stage. A representative block for each case was selected for immunohistochemical analysis, with CD68 and CD163 markers. The primary antibodies anti-CD163 (Rabbit Monoclonal clone EPR4521 dilution 1:250, Epitomics, Burlingame, CA, USA) and anti-CD68 (Mouse Monoclonal clone PGM-1 dilution 1:60, Dako, Carpinteria, CA, USA) were, respectively, placed on the same slide to make a double staining and incubated according to the protocol IHC DS uDAB-uRED. It was used as chromogen diaminobenzidine (DAB) for CD163 and FAST RED for CD68. When the tissue sections were counterstained with Mayer's hematoxylin. Sections of strongly positive spleen (CD163) and tonsil (CD68) were used as positive controls. Negative control was performed by substituting the primary antibody with a non-immune serum at the same concentration. The control sections were treated in parallel with the samples.

Evaluation of immunohistochemical staining

The stains for CD68 and CD163 were considered positive when there was a strong granular cytoplasmic or cytoplasmic and membrane staining patterns in cells of monocyte/macrophages lineage.

Slide grading

Macrophages were quantified by systematically screening the entire carcinoma area at low magnification using a $\times2.5$ or $\times5$ lens and selecting the areas with highest density of macrophages and by counting the macrophages M1 and M2 in three hot spots using power $\times40$ lens. Finally, the mean of both the number of M1 macrophages and M2 macrophages in these three hot spots was obtained. All counting was performed by one investigator (MRR) unaware of clinical data.

Statistical analysis

Data are presented as means $\pm$ s.d. from at least three independent experiments. Statistical analysis of the data was performed by Student's *t*-test. *P* values of $\leqslant0.05$ were considered statistically significant.

ABBREVIATIONS

CAFs, cancer-associated fibroblasts; CAMs, cancer-associated macrophages; CM, conditioned media; COX-2, cycloxigenase-2; EMT, epithelial–mesenchymal transition; EPCs, endothelial progenitor cells; HPFs, human prostate fibroblasts; HUVEC, human umbilical vein endothelial cells; IFN-γ, interferon gamma; IL, interleukin; LPS, lipopolysaccharides; MCP-1, monocyte chemotactic protein-1; M-CSF, macrophage colony-stimulating factor;

PCa, prostate carcinoma; ROS, reactive oxygen species; SDF-1, stromal-derived growth factor-1; SMA, α-smooth muscle actin; TGF-β, transforming growth factor-β.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

This work was supported by the Associazione Italiana Ricerca sul Cancro (AIRC), by Istituto Toscano Tumori, and by MIUR (PRIN 2008). Pedro Barcellos-de-Souza was supported by postdoctoral fellowship from Capes (Ministério da Educaçao, Brazil).

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Supplementary Information accompanies this paper on the Oncogene website (http://www.nature.com/onc)