The supernatant of cervical carcinoma cells lines induces a decrease in phosphorylation of STAT-1 and NF-κB transcription factors associated with changes in profiles of cytokines and growth factors in macrophages derived from U937 cells

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Abstract

Macrophages are present in the tumor microenvironment and acquire different phenotypic and functional characteristics in response to microenvironmental signals. Macrophages can be differentiated into two phenotypes: M1 or pro-inflammatory (classically activated), and M2 or anti-inflammatory macrophage (alternatively activated). In response to the microenvironment, macrophages activate transcription factors as STAT1 and NF-κB-p65 for M1 macrophages or STAT3 and STAT6 for M2 macrophages; activation impacts on the profile of cytokine, chemokines and growth factors secreted by macrophages. We evaluated the effect of the supernatant of cervical-derived carcinoma cell lines HeLa, SiHa, and C-33A on the phosphorylation of transcriptional factors STAT1, NF-κB-p65, and STAT6, and their impact in the profile of secretion of cytokines and growth factors by macrophages derived from the U937 cell line. The results show that in macrophages, these supernatants induce a decrease in the phosphorylation of NF-κB-p65 and STAT1 in U937-macrophages accompanied by an increase in the secretion of IL-10, IL-6, MCP-1, and IL-8, as well as GM-CSF, G-CSF, PDGF-AA, PDGF-BB, and VEGF. Our results suggest that HeLa, SiHa, and C-33A cell lines down-regulate the activation of transcription factors characteristic of M1 macrophages (STAT1, NF-κB-p65) and induce the secretion of factors that favor tumor growth.

Keywords

Cervical cancer cell lines, cytokines, growth factors, macrophages, transcription factors

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Introduction

The tumor microenvironment is key to understanding how it contributes significantly to cancer progression. Tumor cells are in direct contact with other cells, such as fibroblasts and endothelial and immune system cells. Various immune effector cells with anti-tumor functions, such as dendritic cells (DC), NK cells, macrophages, and CD8+ T lymphocytes, can be recruited to the tumor microenvironment. However, over time, via secretion of soluble molecules such as growth factors and cytokines by the tumor cells, as well as mechanisms of direct contact between tumor cells and the immune system, cells are able to educate immune cells in order to minimize or turn off their anti-tumor functions, which finally promotes tumor growth. Macrophages in the tumor microenvironment are called tumor-associated macrophages (TAM) and constitute a significant part of the leukocytic infiltrate in this microenvironment. The role of macrophages here is controversial; however, there is a tendency for these cells to be related with poor prognosis in several cancer types, including cervical cancer (CC). During tumor progression, circulating monocytes are recruited into the tumor in response to growth factors such as Colony-stimulating Factor-1 (CSF-1), Vascular Endothelial Growth Factor (VEGF), and Monocyte Chemotactic Protein-1 (CCL2/MCP-1), CCL-3, -4, -5, and -8 chemokines, as well as other molecules. Once they have migrated into the tumor mass, monocytes are differentiated into macrophages, which exhibit different phenotypical and functional characteristics in response to microenvironmental signals generated by tumor and stromal cells. In general, macrophages can be divided into two phenotypes with opposite functions.

Classically activated macrophages (M1 macrophages) can be induced by IFN-γ and microbial components such as LPS; these signals dictate a transcriptional response in macrophages that shapes the phenotype and function of these cells. M1 macrophages are controlled mostly by Signal Transducers and Activators of Transcription 1 (STAT1), as well as NF-κB. In response to the activation of these factors a secretion high of pro-inflammatory cytokines such as IL-1, TNF-α, IL-12, IL-6, IL-23, as well as Reactive Oxygen Species (ROS), and nitrogen intermediates are induced, which can induce death in cancer cells.

In contrast, alternatively activated macrophages (M2 macrophages) can be induced by distinct stimuli, and are additionally classified as M2a, M2b, and M2c. M2 macrophage responses are triggered primarily through activation of STAT3 and STAT6; these macrophages are characterized by low secretion of pro-inflammatory cytokines, and instead, they express markers related with immune-suppressive phenotypes such as Arg-1, Ym1, FIZZ1, the mannose (CD206) and scavenger receptors (CD163), IL-10, and chemokines such as CCL-17, CCL-22, and CCL-24. M2 macrophages release a variety of growth factors, such as VEGF, Epidermal Growth Factor (EGF), and Fibroblast Growth Factor (FGF); factors that, in a tumor environment, provide a nutritional advantage for cancer cells. Cancer cells secrete various soluble molecules that can promote their growth and differentiation, and, at the same time, can modulate the immune response to create an optimal environment for the tumor. Among the cytokines and growth factors delivered by tumor cells that favor differentiation into M2 macrophages are IL-10, CCL chemokines, VEGF, and Platelet-Derived Growth Factor (PDGF), among others. CC remains one of the most common cancer types among women in developing regions.

Materials and methods

Cell culture

Cervical derived carcinoma cell lines HeLa (HPV-18-positive), SiHa (HPV-16-positive), and C-33A (HPV-negative) were kindly provided by Petra Boukamp PhD (DKFZ, Heidelberg, Germany). Human leukemic monocyte-lymphoma cell line U937 was obtained from the American Type Culture Collection (ATCC CRL- 1593.2TM). Cervical derived carcinoma cell lines were grown in DMEM containing 10% heat-inactivated FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin (all from Gibco Invitrogen Corp., Carlsbad, CA, USA). U937 cells were grown in Roswell Park Memorial Institute (RPMI)-1640 culture medium supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin (all from Gibco Invitrogen Corp.). These media will...
be referred to as DMEM-S and RPMI-S; respectively. All cell lines were incubated at 37°C in a humidified atmosphere with 5% of CO₂.

**Supernatant of cervical derived carcinoma cell lines HeLa, SiHa, and C-33A**

Cervical derived carcinoma cell lines HeLa, SiHa, and C-33A were grown in 75 cm² flasks until reaching 80–90% confluence. Cells were harvested using trypsin-EDTA solution (GIBCO® Life Technologies Corp.). A total of 250,000 cells were plated into a 25 cm² flask containing 6 ml of DMEM-S. Cultures were maintained at 37°C in a humidified atmosphere with 5% of CO₂; after 5 d of incubation the supernatant was collected under sterile conditions, centrifuged at 400 g for 10 min, and stored at –80°C until use.

**Differentiation and activation of the U937 monocyte cell line toward the macrophage**

For complete differentiation of U937 cells into macrophages, 1 x 10⁶ U937 cells were plated into 12-well tissue culture plates containing 2 ml of RPMI-S at a final concentration of 200 nM of Phorbol Myristate Acetate (PMA) and incubated for 3 d. Adherent cells were washed three times with PBS. These cells will be referred from now as M0 macrophages.

Activation of the macrophages was achieved by incubating M0 macrophages previously differentiated in the presence of 100 ng/ml of LPS for 24 h. After this, the medium containing LPS was removed completely, and cells were washed with PBS. These cells are referred to as M1 macrophages.

 Likewise, M0 macrophages were treated with 20 ng/ml of IL-10 (recombinant human IL-10, BioLegend, San Diego, CA, USA) for 24 h, after that the cells were washed. These cells are referred to as M2 macrophages.

All incubations were at 37°C in a humidified atmosphere with 5% of CO₂.

**Culture of macrophages in conditioned medium containing supernatants of cervical derived carcinoma cell lines**

Previously obtained M0 and M1 macrophages were cultured in a total volume of 2 ml of RPMI-S containing 30% of the supernatant of HeLa, SiHa, or C-33A cell lines for 1 h; subsequently, the cells were harvested with Accutase (StemPro® Accutase; Thermo Fisher Scientific, Waltham, MA, USA) and stained for assessment of the phosphorylation of transcription factors (STAT1, STAT6, and NF-κB-p65) by Flow Cytometry (FC).

For assessment of cytokines and growth factors, M0 macrophages were cultured in RPMI-S containing 30% of the supernatants of HeLa, SiHa, or C-33A cell lines or for 3 and 5 d at 37°C in a humidified atmosphere with 5% of CO₂. We used M1 and M2 macrophages like experimental controls of this assays. Afterward, the supernatant was collected under sterile conditions, centrifuged at 400 g for 10 min, and stored at –80°C until determination of cytokines and growth factors.

**Cytokine and growth factor quantification by flow cytometry**

Bead-based multiplex assays were employed to quantify cytokines (LEGENDplex™ Human Inflammation Panel) and growth factors (LEGENDplex™ Human Growth Factor Panel; both kits from BioLegend, Inc., San Diego, CA, USA) in supernatants of cervical derived carcinoma cell lines and in the supernatant from macrophages incubated or not with tumor cell supernatant.

Briefly, 25 μl of thawed supernatants, diluted standard, and blanks were added into the corresponding tubes; 25 μl of pre-mixed beads and detection Abs were added to all of the tubes. Then, the tubes were incubated for 2 h at room temperature with shaking. After this, and without washing, 25 μl of StreptAvidin-Phycocerythrin (SA-PE) conjugate was added, and the tubes were incubated for 30 min and finally washed and suspended in 200 μl of wash buffer. Data were acquired in an Attune Acoustic Focusing Cytometer (Life Technologies, Carlsbad, CA, USA). A total of 4000 and 2000 events were acquired for inflammation panel or growth factor panel; respectively. The files were analyzed utilizing BioLegend LEGENDplex™ Data Analysis Software. Results represent the concentration expressed in pg/mL.

**TGF-β1 and PGE-2 quantification in the supernatants of cervical derived carcinoma cell lines HeLa, SiHa, and C-33A**

Identification of TGF-β1 and PGE-2 was performed by ELISA using TGF-β1 immunoassay and Prostaglandin E2 Assay Kits (both R&D Systems), respectively. The assay was made following the manufacturer’s instructions. The OD was determined using a microplate reader (SynergyTM HT Multi-Mode Microplate Reader; Bio Tek Instruments, Inc., Winooski, VT, USA) at 450 nm (wavelength correction at 540 nm). Results represent the concentration expressed in pg/mL.

**Antibodies**

Fluorochrome-labeled monoclonal primary Abs for staining were purchased from BD Biosciences.
Perm Buffer III (BD Biosciences, San Jose, CA, USA), and the following were utilized: PE anti-human STAT1-(N-terminal) (Clone 1/Stat1); PE anti-human STAT1 (pY701) (Clone 4a); PE anti-human STAT6 (Clone 23/Stat6); PE anti-human STAT6 (pY641) (Clone 18), and Alexa-Fluor 647 anti-human NF-κB-p65 (pS532) (Clone K10-895.12.50).

Assessment of transcription factors STAT1, STAT6, and NF-κB-p65 by FC

Expression transcription factors in M0 and M1 macrophages treated or not for 1 h with the supernatant of cervical-derived carcinoma cell lines HeLa, SiHa, or C-33A were assessed by FC analysis. Briefly, M0 or M1 macrophages in assay tubes were stained with LIVE/DEAD viability stain (Life Technologies Corp.) for 30 min to discriminate between viable and non-viable cells. Afterward, cells were washed first with PBS and then with stain buffer (FBS) (BD Pharmingen, San Jose, CA, USA), re-suspended by vortexing in 250 μl of Fixation buffer (BD™ Phosflow) and immediately incubated for 20 min at 4°C. Fixed cells were washed with stain buffer and immediately permeabilized, by slowly adding and under vortex conditions, the cold Perm Buffer III (BD™ Phosflow). Cells were incubated on ice for 30 min and then washed with 2 ml of stain buffer. Cells were re-suspended in 100 μl of stain buffer and were stained with the corresponding Abs for 30 min at 4°C. Afterwards, the incubated cells were washed and re-suspended in stain buffer for analysis by FC. Attune Acoustic Focusing Cytometer (Life Technologies, Carlsbad, CA, USA) was utilized to acquire 20,000 events in the region of viable cells. Data were processed with FlowJo ver. 10.0.8 statistical software (Tree Star, Inc., Ashland, OR, USA). Results are reported as percentage of expression or as the geometric Mean Fluorescence Intensity (MFI).

Statistical analysis

The data obtained are shown as means ± SD of the values of three independent experiments. Comparisons among groups were performed with Student t-test. Only values of P < 0.05 were considered significant.

Results

Secretion of MCP-1, IL-6, and IL-8 by cervical derived carcinoma cell lines HeLa, SiHa, and C-33A

We determined in the supernatant of HeLa, SiHa (HPV-18 and -16-positive; respectively) and C-33A (HPV-negative) cell lines, the profile of secretion of cytokines and growth factors using bead-based multiplex kits. The concentrations of cytokines and growth factors for each supernatant are depicted in Figure 1A, where heatmaps are depicted representing changes in the profile of cytokines (left) and growth factors (right) secreted by HeLa, SiHa, and C-33A cervical derived carcinoma cell lines in their respective supernatants. Interestingly, MCP-1, IL-6, and IL-8 were secreted by these cancer cells (Figure 1B), HeLa and SiHa released higher amounts of these cytokines compared with C-33A (all comparisons with a significant difference of *p < 0.001).

Release of VEGF, PDGF-AA, PDGF-BB, HGF, EPO, Ang-2, G-CSF as well as TGF-β1 and PGE-2 by HeLa, SiHa, and C-33A cell lines

The concentrations of growth factors released by cervical derived carcinoma cell lines are presented in Figure 1C. In general, we observed that VEGF, PDGF-BB, HGF, Ang-2, and G-CSF were secreted in high amounts in all CC cells. It is important to highlight that EPO and PDGF-BB were found in high quantities in HeLa and SiHa supernatants in comparison with C-33A supernatant (◆p < 0.05).

On the other hand, in C-33A supernatant, were found HGF, VEGF, Ang-2, and PDGF-AA in elevated concentrations compared with the HeLa and SiHa supernatants (◆p < 0.05). In relation to TGF-β1 and PGE-2 secreted by cervical derived carcinoma cell lines, we found that HeLa is the primary cell line that produces these intermediaries in relation to SiHa that produces a smaller amount of these two factors or C-33A that only release PGE-2 in a low proportion (*p < 0.001) HeLa vs SiHa and C33-A.

Effect of HeLa, SiHa, and C-33A supernatants in cytokine production by macrophages

We also evaluated the effect of supernatant of cervical derived carcinoma cells on the secretion of cytokines by macrophages cultured in the presence of supernatant for 3 and 5 d with respect to M0 (only differentiated with PMA), M1 (differentiated with PMA and activated with LPS), and M2 macrophages (macrophages differentiated with PMA and incubated in the presence of IL-10).

Cytokines were measured in supernatants of macrophages by using the bead-based multiplex assay. The heatmaps in Figure 2A provide evidence about the differential amounts of cytokines secreted by macrophages incubated or not with supernatants for 3 and 5 d; respectively. Figure 2B shows the increase in the secretion of IL-10, IL-6, MCP-1 and IL-8 cytokines at 3 and 5 d compared with M0 and M1 macrophages (*p < 0.01). Another interesting fact was the increase
in the secretion of TNF-α and IL-1β by macrophages cultivated with supernatant of C-33A cervical carcinoma cell line in comparison with all experimental groups in both 3 and 5 d (*p < 0.01; Figure 2C). Finally, macrophages increased the secretion of IFN-α in the presence of supernatants of HeLa, SiHa, and C-33A in comparison to M0, M1, and M2 macrophages (*p < 0.01; Figure 2C).

**Induction of growth factors in macrophages treated with the supernatant of HeLa, SiHa, and C-33A cell lines**

It has been reported that macrophages associated with tumors possess the ability to release growth factors that can contribute to tumor growth. To evaluate the effect of the supernatant of cervical derived carcinoma cell lines on the secretion of growth factors by macrophages, supernatants from macrophages cultivated under the conditions described previously were recollected and analyzed by FC based on a bead-based multiplex assay. In Figure 3A, the heatmaps are shown for growth factors secreted by macrophages at 3 (left) and 5 d (right) of culture for the different experimental groups, in general, it can be observed that macrophages incubated with the different supernatants secreted G-CSF and VEGF.

We observed that, on d 3 of culture the concentration of G-CSF was increased in HeLa, SiHa, and C-33A groups in comparison with M0 and M1 (Figure 3B); in addition, the amount of G-CSF, as well as GM-CSF, were increased by macrophages in the presence of HeLa, SiHa, and C-33A supernatants vs. M0 and M1 groups at d 5 of culture (Figure 3C; *p < 0.05). On the other hand, at d 3, as shown in Figure 3C, the secretion of PDGF-AA was induced.
in macrophages by the effect of the three supernatants in comparison with M0 and M1 macrophages (p < 0.05). At 5 d of culture of the macrophages in their different conditions, we observed an increase of PDGF-BB secretion by effect of HeLa, SiHa, and C-33A supernatants in comparison with M1 (p < 0.05), Figure 3E, while SiHa and C-33A increased the release of PDGF-AA and VEGF compared with M0 and M1 macrophages (p < 0.05). Hela, SiHa, and C-33A supernatants induce a decrease in the phosphorylation of STAT1 and NF-kB-p65

Macrophage responds to different stimuli of the microenvironment that affects the profile of phosphorylation of its transcriptional factors, which will determine the phenotype of secretion and expression of markers corresponding to each phenotype. We evaluated by FC the phosphorylation of NF-kB-p65, STAT1, and STAT6. Our results are presented in Figure 4. We observed that the supernatants of HeLa and C-33A induced a significant decrease in the phosphorylation of NF-kB-p65 in M1 macrophages. Interestingly, HeLa, SiHa, and C-33A supernatants induced a significant decrease in phosphorylated STAT1 in M1 macrophages. Finally, we observed that there was a tendency toward the increase of the percentage of expression of STAT6-P due to the effect of supernatants in macrophages, especially in the case of the HeLa supernatant.

Supernatants of HeLa, SiHa, and C-33A did not induce any effect in these transcription factors in M0 macrophages (data not shown).

Figure 2. HeLa, SiHa, and C-33A supernatants induced the secretion of IL-10, IL-6, MCP-1, and IL-8 by M2 macrophages compared with M0 and M1 macrophages. Macrophages were obtained by differentiation of the U937 cell line with PMA (M0 macrophages), stimulation with LPS (M1 macrophages), treatment with IL-10 (M2 macrophages), or cultivation in the presence of 30% of HeLa, SiHa, or C-33A for 3 and 5 d (see Materials and methods). The supernatant was collected again, and cytokines were quantified using bead-based multiplex assay by flow cytometry. (A) Heatmaps generated by LEGENDplex Data Analysis software, representing the changes in the concentrations of cytokines on d 3 (left) and 5 (right) in each experimental group. (B) and (C) More representative cytokines are shown, based on the corresponding analysis for macrophages cultured in the presence or not of HeLa, SiHa, and C-33A supernatants for 3 and 5 d, respectively. The results of each experimental condition were obtained from assays conducted in triplicate and are represented as the mean ± SD of the concentrations in pg/ml. *p < 0.01 in HeLa, SiHa, and C-33A vs M0 and M1.
Discussion

In response to the tumor microenvironment, macrophages can acquire an immunosuppressive phenotype that promotes growth, cell invasion, neovascularization, and metastases. Our data are derived from macrophages exposed to a microenvironment generated by soluble factors secreted by CC cells lines. First, as a result of the characterization of the tumor cell supernatant, it was interesting to observe that HeLa and SiHa produce a higher concentration of IL-8, MCP-1, and IL-6. These factors could be related to the presence of high-risk HPV, such as HPV-18 and -16, in HeLa and SiHa cell lines, respectively. Expression of IL-8 and MCP-1 at the mRNA and protein levels has been reported in other tumor cells, such as human esophageal carcinoma cell lines, in breast and prostate cancer, in which these have been related positively with the recruitment of immunosuppressive macrophages and angiogenesis. IL-8 is a factor related to tissue injury, fibrosis, and angiogenesis, and also it is considered a growth factor for tumor cells. Additionally, IL-8 has been found in higher concentrations in serum of patients with prostate cancer, hepatocellular carcinoma, and in those patients positive for HPV infection in comparison with healthy controls. MCP-1 is expressed in various types of cancer, and,
in turn, its expression in tumor cells is significantly correlated with the infiltration of TAM in the tumor site. Production of IL-6 has been reported for other human cell lines of liver and prostate carcinoma, among others. IL-6 also has been reported in CC in vitro studies and dysplastic lesions, especially in epithelial cells of high-grade CIN. We previously reported the secretion of IL-6 by CC cell lines, and Heusinkveld et al. demonstrated that IL-6 and PGE-2 secreted by CC cell lines were key factors for inducing M2 macrophages with the suppressor phenotype. Underlining the role of this cytokine in macrophage polarization, it has been found in a model of insulin resistance that IL-6 promotes the M2 phenotype in macrophages, and Chastain et al. demonstrated recently that the treatment of RAW cell macrophages with IL-6 up-regulated the expression of characteristic markers of M2 macrophages, like CD206 and IL-4. It is important to stress that we observed a high secretion of TGF-β1 and PGE-2 by the HeLa carcinoma derived cell line. TGF-β1 can regulate innate and acquired immunity by inducing regulatory T cells, which can suppress T effector cells. This cytokine is able to change NKT cells and macrophages to the regulatory phenotype. In addition to the impact on regulation of the immune system, TGF-β1 and IL-6 act as promoters of Epithelium Mesenchymal Transition (EMT). Taken together, HeLa and SiHa have a secretion profile of cytokine and chemotactic factors that collaborates with establishment and growth of the tumor. Also, its secreted factors cooperate in the infiltration of immune cells as monocytes that upon arrival at the tumor niche are influenced by a microenvironment dominated by cytokines like IL-6, TGF-β1, and PGE-2, which promote a suppressor phenotype. PDGF-BB is an essential component of the angiogenic process since it is involved in the maturation of the vessel inside the tumor microenvironment.

![Flow Cytometry Graphs](image-url)

**Figure 4.** Phosphorylation of STAT1 (Y701) and NF-κB-p65 is decreased in macrophages due to HeLa, SiHa, and C-33A supernatants. Macrophages were obtained by differentiation of the U937 cell line with PMA (M0 macrophages), stimulation with LPS (M1 macrophages), cultured in the presence or not of 30% of HeLa, SiHa, or C-33A for 1 h (see Materials and methods), and harvested and processed by intracellular staining for measuring the expression of NF-κB-p65, STAT1 (Y701), and STAT6 (Y641). Results are shown as % and MFI. Representative flow cytometric graphs of three independent experiments performed in triplicate are shown.
activation of the signaling pathway, induces VEGF secretion in ovarian epithelial carcinoma. In breast cancer and pancreatic cell lines, both factors are considered mitogenic in a paracrine or autocrine loop resulting in tumor growth and, specifically for PDGF, its involvement has been suggested in EMT that leads to tumor growth, angiogenesis, invasion, and metastasis in solid tumors such as CC. C33-A, despite being a cell line negative to HPV infection, was the main cell line secreting a significant amount of HGF and Ang-2, in addition to PDGF-AA and VEGF. The presence of HGF has also been reported in ovarian cancer cells, where it was associated with the mesothelial–mesenchymal transition, one of the key mechanisms in CC, as well as the invasion of mesothelial cells. The HGF/MET complex induced the expression of VEGF and synergized with the VEGF receptor (VEGFR), favoring angiogenesis and lymph-angiogenesis increasing tumor malignancy. The importance of Ang-2 lies in its potential to cooperate with the tumorigenic process related to invasiveness since it is co-overexpressed with some MMP, and is involved in lymph-angiogenesis and angiogenesis acting synergistically with the effects mediated by VEGF. The specific nature of each CC cell can determine a characteristic pattern of concentration of the different cytokines, TGF-β1, PGE-2, and growth factors measured. Practically all factors secreted significantly by the cell lines are involved in autocrine signaling to promote their growth and proliferation. These factors are closely related to tumorigenic processes such as angiogenesis, and lymph-angiogenesis. The improvement of the invasive capacity towards surrounding tissues establishes new tumor niches and immunosuppression.

Here, the effect of supernatants from CC cells on the phosphorylation of transcriptional factors and their effect on U937 macrophages regarding their secretion profile of cytokines and growth factors was evaluated. This cell line has been a widely used model to elucidate a variety of biological mechanisms related to monocyte and macrophage functions. In this sense, it is important to stress that the supernatants from CC HeLa, SiHa, and C-33A cells induced in macrophages an increase of the secretion of IL-10, IL-6, MCP-1 and IL-8, as well as of GM-CSF, G-CSF, PDGF-AA, PDGF-BB, and VEGF. Moreover, it is important to mention that IL-10 is an anti-inflammatory and immunosuppressive cytokine that can suppress the cytotoxic activity of T and NK cells, down-regulate Ag presentation, and prevent the maturation of DC in situ, favoring macrophage differentiation. IL-6 directly effects angiogenesis since this contributes to vascularization in ovarian cancer, but also can induce the release of VEGF from CC and glioblastoma cells and other inflammatory cytokines with angiogenic power, such as IL-8. Angiogenesis represents an essential process for the successful establishment and growth of tumor cells. Moreover, angiogenic factors as VEGF and IL-8 were released by macrophages treated with the supernatants of HeLa, SiHa, and C-33A; these could be factors induced in response to IL-6. In addition to angiogenesis, IL-8 favors the infiltration of monocytes and macrophages. In a model of human skin carcinoma utilizing the HaCaT cell line, IL-6 also regulated MCP-1 and GM-CSF, which promoted the proliferation and invasiveness of a benign skin tumor to an invasive and well vascularized by the increase of IL-8 and VEGF.

MCP-1 expression and macrophage infiltration have been correlated with poor prognosis and metastasis in human breast cancer. Any augmentation of secretion of MCP-1 by macrophages in response to supernatants of HeLa, SiHa, and C-33A cells could result in a poorly immunogenic microenvironment where tumor cells can grow successfully. The increased release of GM-CSF and G-CSF by macrophages treated with supernatants could favor the growth and establishment of these cells with immunosuppressive activity through an autocrine response since G-CSF could act in synergy with IL-6 via STAT3, favoring the M2 phenotype in the macrophage.

Regarding PDGF, it has been shown that a higher concentration of PDGF in TAM is directly related to tumor growth in the healthy lung tissue surrounding the periphery of the tumor. Moreover, in breast cancer, PDGF-BB released by TAM can activate fibroblasts and osteoclasts in order to support cancer stem-cell self-renewal.

HeLa, SiHa, and C-33A supernatants were able to induce an essential decrease in STAT1 phosphorylation as well as of NF-κB-p65 in activated M1 macrophages. Dys-regulation of NF-κB and STAT1 could comprise a mechanism through which an anti-tumor response is reduced by macrophages, because many cytokines and molecules with antitumor activity are secreted in response to the activation of NF-κB and STAT1. Likewise, we observed that the supernatant of HeLa cells induced phosphorylation in STAT6. Taken together, all of this suggested that HeLa, SiHa, and C-33A induce several effects in the macrophage, the main one being disruption of STAT1 phosphorylation, which is an essential pathway for the macrophage to perform anti-tumor and immune responses.

Conclusions

The CC cell lines HeLa, SiHa, and C-33A release cytokines and growth factors that can de-regulate immune responses and also induce growth,
immunosuppression, and angiogenic pathway activation. HeLa, SiHa, and C-33A supernatants induced a decrease in STAT1 and p65 phosphorylation in macrophages. Likewise, the supernatants induced the secretion of growth factors, some with angiogenic potential, providing advantages for successful tumor growth.

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