



Regulation of immunophenotype modulation of monocytes-macrophages from M1 into M2 by prostate cancer cell-culture supernatant via transcription factor STAT3

R. Solís-Martínez^a, M. Cancino-Marentes^b, G. Hernández-Flores^c, P. Ortiz-Lazareno^c,
G. Mandujano-Álvarez^d, C. Cruz-Gálvez^b, E. Sierra-Díaz^e, C. Rodríguez-Padilla^f, L.F. Jave-Suárez^c,
A. Aguilar-Lemarro^c, A. Bravo-Cuellar^{c,g,*}

^a Programa de Doctorado en Ciencias Biomédicas Orientación Inmunología, Centro Universitario de Ciencias de la Salud (CUCS), Universidad de Guadalajara, CP 44340 Guadalajara, Jal., Mexico

^b Programa de Doctorado en Farmacología, Centro Universitario de Ciencias de la Salud (CUCS), Universidad de Guadalajara, CP 44340 Guadalajara, Jal., Mexico

^c División de Inmunología, Centro de Investigación Biomédica de Occidente, Instituto Mexicano del Seguro Social (IMSS), Sierra Mojada 800, Col. Independencia, CP 44340 Guadalajara, Jal., Mexico

^d Hospital Médica Tabasco, DACS, UJAT, Av. Paseo Tabasco 808, CP 86040 Villahermosa, Tabasco, México

^e Servicio de Urología, Centro Médico Nacional de Occidente (CMNO), Instituto Mexicano del Seguro Social (IMSS), CP 44340 Guadalajara, Jal., Mexico

^f Departamento de Virología e Inmunología, Universidad Autónoma de Nuevo León. Av. Pedro de Alba S/N, Ciudad Universitaria, Apartado Postal 46-F, CP 66450 San Nicolás de los Garza, Nuevo León, Mexico

^g Centro Universitario de los Altos (CUALTOS), Universidad de Guadalajara, CP 47600 Tepatlán de Morelos, Jal., Mexico

ARTICLE INFO

Keywords:

M1
M2
STAT3
Prostate cancer
Stattic™

ABSTRACT

Background: Transcription factor STAT3 has a prominent innate immunity effect on cancer progression. We determined the regulation of STAT3 in the immunophenotype modulation of macrophages from M1 into M2 induced by the cell-culture supernatant of the Prostate-Cancer line PC3.

Methods: Monocytes-macrophages from healthy donors were cultured in the supernatant of PC3 cells, membrane proteins, and intracytoplasmic and phosphorylated STAT3 were measured using flow cytometry, while cytokines and growth factors were studied using luminescence. Cytotoxicity and nitric oxide were evaluated via colorimetric assays.

Results: The supernatant of PC3 prostate-tumor cells effectively induced macrophages toward an M2 profile, and the expression of phosphorylated STAT3 in the monocytes-macrophages notably increased, and mainly related to IL-10. In the group of monocytes-macrophages treated with a STAT3 inhibitor, the macrophages were induced toward an M1 phenotype.

Conclusions: In this study, we showed that the secretion profile of PC3 prostate-cancer cells induces a change in macrophage phenotype from M1 into M2, and that the phenomenon is related to phosphorylation of transcription factor STAT3 and IL-10.

1. Introduction

Prostate cancer is an important public health problem because it is the most frequently diagnosed type of cancer and is the second most common cause of cancer-related death in men worldwide. In 2016, there were approximately 180,890 new prostate-cancer cases and approximately 26,120 deaths in the United States [1]. Also, morbidity and mortality rates related to prostate cancer are increasing as a result of the increasing age of the population worldwide.

Today, the cancer microenvironment is known to comprise tumor and stromal cells and involves a complex network of reciprocal

interactions between tumor cells and inflammatory cells. In prostatic tumors, histological data have revealed the presence of CD4+T cells, CD8+T cells, Natural Killer (NK) cells, Dendritic Cells (DC), and macrophages [2,3], and a dense infiltration of Tumor-Associated Macrophages (TAM) has been observed in both epithelial and stromal compartments. These observations are important because the patients prognosis can differ depending on the tumor type and the predominant type of the immune cells present in the tumor [4]. In some tumors, such as colon, breast, and lung tumors, as well as in prostate cancer, the presence of tumor-infiltrating lymphocytes is associated with good prognosis, and the presence of TAMs is associated with poor prognosis

* Corresponding author at: Centro de Investigación Biomédica de Occidente, IMSS, Sierra Mojada 800, Col. Independencia, 44340 Guadalajara, Jal., México.
E-mail address: abravoc@prodigy.net.mx (A. Bravo-Cuellar).

<https://doi.org/10.1016/j.imlet.2018.02.009>

Received 1 August 2017; Received in revised form 1 February 2018; Accepted 20 February 2018

Available online 23 February 2018

0165-2478/ © 2018 Published by Elsevier B.V. on behalf of European Federation of Immunological Societies.

[5–7].

Macrophages play an indispensable role in innate and acquired immunity against foreign pathogens and tumor cells. However, there is evidence that the tumor microenvironment can negatively modulate the behavior of TAM, favoring tumor progression, including cancer-cell growth and spread, angiogenesis, and immune suppression [8,9]. This effect can be understood via the functional plasticity of the macrophages, which can be broadly classified as either classically activated macrophages (M1 macrophages) or alternatively activated macrophages (M2 macrophages), according to their functions [10]. M1 macrophages are potent effector cells that kill microorganisms and tumor cells and produce pro-inflammatory cytokines, such as Interferon gamma (IFN- γ), Tumor Necrosis Factor alpha (TNF- α), and Interleukin (IL)-2. The M1 phenotype is characterized by its capacity to present antigens and increase IL-12 and IL-23 production with subsequent activation of a polarized type-I response. In contrast, M2 macrophages reduce these inflammatory and adaptive Th1 responses by producing anti-inflammatory factors, such as IL-10 and Transforming Growth Factor Beta (TGF- β), and promoting angiogenesis, tissue remodeling, and repair [11,12]. Macrophages are dynamic cells and can switch from an activated M1 type back into an M2 state, and vice versa, upon the induction of specific signals [13,14].

M2-phenotype macrophages are a significant component of inflammatory infiltrates in prostate cancer. In men treated with primary androgen- deprivation therapy, increased M2-phenotype levels in a biopsy are predictive of recurrence and decreased survival. Polarization to an M2 phenotype can be induced by different interleukins such as IL-4, IL-10, and IL-13, and IL-22 [15] activating signal pathways, such as signal transducer and activator of transcription 3 (STAT3) [16,17].

STAT3 is a member of the STAT family of transcription factors that possess critical functions in development, cell growth, and homeostasis in a variety of tissues. STAT3 can be activated by many cytokines and growth-factor receptors through the phosphorylation of specific residues (Y705, Ser727). In normal cells, STAT3 activation is tightly regulated and transient due to its importance in modulating a variety of biological functions through the activation of the transcription of a diverse set of genes, such as *VEGF*, *BCL-2*, *BCL-XL*, and *C-MYC*. Furthermore, STAT3 plays an important role in maintaining the pluripotency and self-renewal ability in embryonic stem cells [18].

Conversely, persistent activation of STAT3 protein in neoplastic cells is detected at a high frequency in many human tumors, including leukemias, lymphomas, multiple myeloma, head and neck cancer, and breast and prostate cancer. Moreover, constitutively activated STAT3 is sufficient to induce some of the properties of transformed cells, including tumorigenicity. Together, these findings provide evidence that STAT3 signaling participates in regulating the processes of cell growth and survival during the oncogenesis of prostate cancer. STAT3 has become an excellent study target for cancer therapy [19] due to its oncogenic characteristics, such as apoptosis inhibition, cell- proliferation promotion, immunosuppression, and angiogenesis. These characteristics can be specifically inhibited by Stattic™ (6-nitrobenzo[b]thiophene-1, 1-dioxide), a small molecule shown to inhibit STAT3 activation and nuclear translocation by preventing the binding of tyrosine-phosphorylated peptide motifs within the STAT3 SH2 domain, facilitating its study [20].

Tumor cells secrete several products that can affect the immune response, and modulation of the macrophage immunophenotype from M1 into M2 by the microenvironment is not well understood. Thus, in this study, we investigated the regulation of STAT3 in the immunophenotypic modulation of macrophages from M1 into M2 generated by the cell-culture supernatant of the Prostate-Cancer cell line PC3.

2. Materials and methods

2.1. Culture reagents

Cells were cultured in either F-12K culture medium (Kaighn's

modification of Ham's F-12 medium; GIBCO™ Invitrogen Corp., Carlsbad, CA, USA) or Roswell Park Memorial Institute-1640 culture medium (RPMI-1640; GIBCO™ Invitrogen Corp.); both media were supplemented with 10 Fetal Bovine Serum (FBS; GIBCO™ Invitrogen Corp.), L-glutamine (at a final concentration of 2 mM; GIBCO™ Invitrogen Corp.), and antibiotics (Penicillin/Streptomycin; GIBCO™ Invitrogen Corp.). These media will be referred to as F-12KS and RPMI-S, respectively.

2.2. Obtaining human monocyte-derived macrophages

Peripheral blood was collected in BD Vacutainer™ tubes with EDTA anticoagulant from 25 healthy male university-student volunteers. Each volunteer was between 25 and 30 years old and signed a letter of informed consent, and clinical charts, thorax radiographies, and general laboratory studies were obtained for each volunteer. Peripheral Blood Mononuclear Cells (PBMC) were isolated using standard density gradient centrifugation in Ficoll (30 min, 2000 rpm, 21 °C; Histopaque™, Sigma-Aldrich™, density = 1.077 g/mL). PBMC were washed three times with RPMI-S, and each wash was followed by centrifugation for 10 min at 1500, 1200 and 1000 rpm, respectively, to reduce platelet contamination. The percentage of macrophages was estimated based on smears prepared by cytocentrifugation dyed with α -naphthyl-acetate esterase for identification and Giemsa for differential counts before and after cell adherence.

PBMC were resuspended in RPMI-S containing 1×10^6 macrophages/6 wells in flat-bottomed plates and incubated at 37 °C in a humidified atmosphere containing 5% CO₂ for 2 h to allow monocyte adherence. After incubation, nonattached cells in each experimental group were removed by aspiration, and the adhered cells were washed with RPMI-S at 37 °C three times. Over 95% of the adhered cells had the staining properties and morphological characteristics of monocytes. The monocyte-macrophage cells were maintained in *in-vitro* culture in RPMI-S and, in all cases, viability was confirmed by Trypan blue staining before the experiments (> 95%).

2.3. Cell lines

Prostate-Cancer PC3 cells (ATCC CRL 1435) were maintained *in vitro*, and passages were performed every 3 days in the F-12KS culture medium. Cells were incubated at 37 °C in a humidified atmosphere containing 5% CO₂.

2.4. Collection of the PC3 prostate cancer cell-line supernatant

Prostate-cancer PC3 cells were grown in a flask to 80–90% confluence and harvested with Accutase (Invitrogen™ by Thermo Fisher Scientific; cat. 00-4555). Then, 1×10^5 PC3 cells were plated in 2 mL of F-12K medium on six-well culture plates. Cells were incubated at 37 °C in a humidified atmosphere containing 5% CO₂ for 4 days. Then, the cell supernatants were collected and stored at –80 °C for less than 8 days.

2.5. Experimental groups

All groups contained 1×10^6 live macrophages suspended in 1 mL of RPMI-S. In a group, macrophages were cultured for 48 h exclusively with the supernatant of PC3 cells at a final concentration of 30% of the total volume, as previously reported. Other cells were incubated for 1 h with a STAT3 inhibitor (Stat3 inhibitor V, Stattic™; ChemCruz™Biochemical sc-202818; Dallas, TX, USA) at a final concentration of 50 μ M. Once completed, the medium with Stattic™ was removed, and the cells were washed three times with RPMI-S culture medium at 37 °C and resuspended in the same medium in the presence of 30% v/v of PC3-cell supernatant and cultured for 48 h.

In other experiments, as previously described, 2×10^6 monocytes

were adhered onto 6-well plates and incubated separately by a Transwell insert (Transwell™; 0.4 μM pores; cat. 3412) with PC3 tumor cells (1×10^6 cells) in F-12KS culture medium either in the presence or not of Stattic™ inhibitor. After 48 h of incubation, the inserts were retired, and macrophages were detached using accutase, with viability 95% by Trypan blue exclusion.

The group of M2-polarized macrophages was obtained by adding 20 ng/mL IL-4 (cat. 204 IL; R&D™ Systems, Inc., L; Minneapolis, MN, USA) and IL-10 (cat. 217-IL; R&D™ Systems, Inc.) to macrophage cultures; for the M1-polarized macrophages, the cultured cells were stimulated with 20 ng/mL of IFN-γ (cat. 285-IF; R&D™ Systems, Inc.) and LipoPolySaccharides (LPS of *Escherichia coli* 055: B5, Sigma-Aldrich™, 100 ng/mL). Cells without treatments were used as negative controls. Then, the supernatants were collected and stored at -20°C until use, and the macrophages were harvested with the help of a scraper. Under the same experimental conditions, the participation was scrutinized of interleukins, adding antibodies against IL-10 (R&D™ Systems, Inc; cat. AF-217-NA; 50 μg/mL; Minneapolis, MN, USA), and IL-6 (BioLegend™; cat. 501109; 40 μg/mL; San Diego, CA, USA), IL-4 (R&D™ Systems, Inc.; cat. MAB204; 50 μg/mL), respectively, or its combination, as recommended by the manufacturer, to macrophages cultured with PC3 supernatant. In all of the experiments, we confirmed cell viability using Trypan blue staining (exclusion > 95%).

2.6. Cytotoxicity assay

The specific lactic acid release assay was performed according to the manufacturer's instructions. Macrophage monolayers were prepared as described previously. In flat-bottomed, 96-well plastic microtiter plates (SDL™), and in triplicate, different Effector:Target (E:T) cell ratios were incubated with 10×10^4 target PC3 cells in 0.2 mL (final volume) of RPMI-S for 18 h at 37°C in a humidified atmosphere containing 5% CO_2 . The plates were centrifuged, and lactic-acid levels were quantified in 0.1 mL of the supernatant of each well utilizing a colorimetric assay (Architect™ i2000 Immunoassay Analyzer; Abbott™ Diagnostics Laboratories, Chicago, IL, USA) at a wavelength of 340 nm.

The spontaneous and total release of lactic acid was determined either by incubating target cells alone in RPMI-S or in sterile distilled water (J. T. Baker™), respectively. The following equation was employed to calculate the percentage of specific cytotoxicity:

$$\text{Specific cytotoxicity(\%)} = \frac{\text{Experimental group lactic acid release} - \text{Spontaneous lactic acid release}}{\text{Total lactic acid release} - \text{Spontaneous release of lactic acid}} \times 100$$

Spontaneous release by target cells did not exceed 15% of the total lactic-acid concentration, and the experiment was repeated three times. The results represent the mean \pm Standard Deviation (SD) of the values obtained and are expressed as the specific percentage of lactic acid released.

2.7. Measurement of nitric oxide via colorimetric assay

The supernatant solutions from all experimental groups were obtained under the same experimental conditions to determine nitric oxide concentrations in colorimetric assays (Cat. KGE-001; Total Nitric Oxide and Nitrate/Nitrite; R&D Systems). The assays were performed as recommended by the manufacturer. Briefly, for the nitrite assay, 50 μL of the sample from the experimental or control groups was combined in each well with reaction diluent and Griess reagents I and II. After 10 min of incubation, the Optical Density (OD) was measured utilizing a microplate reader (Synergy HT Multi-Mode Microplate Reader; BioTek Instruments, Inc., Winooski, VT, USA) at 540 nm (wavelength correction at 690 nm). For the nitrate reduction assay, 50 μL of the samples from the experimental and control groups was mixed with NADH and nitrate reductase and, after incubation for 30 min at room temperature, Griess reagents I and II were added. Finally, after 10 min at room

temperature, the OD was determined at 540 nm (wavelength correction at 690 nm) as recommended by the manufacturer. The experiments were repeated three times independently, and the results are expressed in μmol/L.

2.8. Assessment of intracytoplasmic and membrane proteins using flow cytometry

The effect of the PC3 prostate cancer-cell supernatant on macrophage polarization was evaluated using two different conditions. Macrophages were cultured with 30% v/v PC3 prostate cancer-cell supernatant for 48 h (MØ + sPC3). Another group of macrophages was cultured for 1 h with 50 μM of STAT3 inhibitor (Stattic™). Then the medium with Stattic™ was removed, and the cells were cultured for another 48 h in the presence of 30% v/v PC3-cell supernatant (MØ + sPC3 + Stattic™). The macrophages were also cultured without any stimuli as a negative control. After these treatments, all groups were incubated with culture medium alone for an additional 48 h.

The expression of CD163, CD206, CD11a, CD45, TNFR1, CD14, CD86, STAT3, and phosphorylated STAT3 (STAT3p) was assessed using flow cytometry. Briefly, all cells in the different experimental groups were detached, washed twice with Phosphate-Buffered Saline (PBS) solution, and resuspended in PBS. Then, we blocked the human Fc Receptors (FcR) using Fc Receptor Blocking Solution (BioLegend™; Human TruStain FcX™, cat. 422302; San Diego, CA, USA) for 10 min before staining with antibodies. Subsequently, the cells were incubated with anti-human CD163-FITC (BioLegend™; FITC anti-human CD163, cat. 333618), anti-human CD206-FITC (BioLegend™; FITC anti-human CD206, cat. 321104), anti-human CD14-PerCP (BioLegend™; PerCP anti-human CD14, cat. 325632), anti-human CD86-PE (BioLegend™; PE/Dazzle™ 594 anti-human CD86, cat. 305434), and anti-human CD11a-PE (BioLegend™; PE anti-human CD11a, cat. 301207), anti-human CD45-PE (BioLegend™; PE anti-human CD45, cat. 368509), and anti-human TNFR1-PE antibodies (BioLegend™; PE anti-human CD120a, clone W15099A, cat. 369903) for 30 min at 4°C . Then, the cells were washed and permeabilized with permeabilization buffer (BioLegend™; Permeabilization Wash Buffer (10X), cat. 421002), and anti-human STAT3p-PE or STAT3-PE antibodies (Santa Cruz, sc-7993 and sc-8019, respectively) were added for 30 min at 4°C . The cells were washed with PBS, fixed with 1% paraformaldehyde, and analyzed with a flow cytometer. An appropriate isotype control and Fluorescence Minus Ones (FMO) were utilized to adjust for background fluorescence, and the results are reported as geometric Mean Fluorescence Intensity (MFI). For each sample prepared in triplicate, at least 20,000 events were acquired in an FC 500™ (Beckman Coulter Life Sciences, Brea CA, USA). Data were processed with FlowJo™ V10-1r5 Single Cell Analyses software (Tree Star, Ashland, OR, USA).

2.9. Cytokine and growth-factor measurements

The levels of cytokines IL-10, IL-4, IL-6, and TNF-α, and TGF-β, Platelet-Derived Growth Factor-A (PDGF-A), Macrophage Colony-Stimulating Factor (M-CSF), Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF), Granulocyte Colony-Stimulating Factor (G-CSF), and Fibroblast Growth Factor (FGF), in all of the experimental and control supernatants described previously, were measured with a Bio-Plex™ 200 System sandwich immunoassay (Bio-Rad, Hercules, CA, USA) and analyzed with Bio-Plex™ Manager ver. 6.0 statistical software according to the manufacturer's instructions. The results are expressed in ng/mL.

2.10. Statistical analysis

All experiments were performed in triplicate, and the results are presented as the mean \pm SD of the values obtained from three independent experiments performed in triplicate. Statistical analysis was

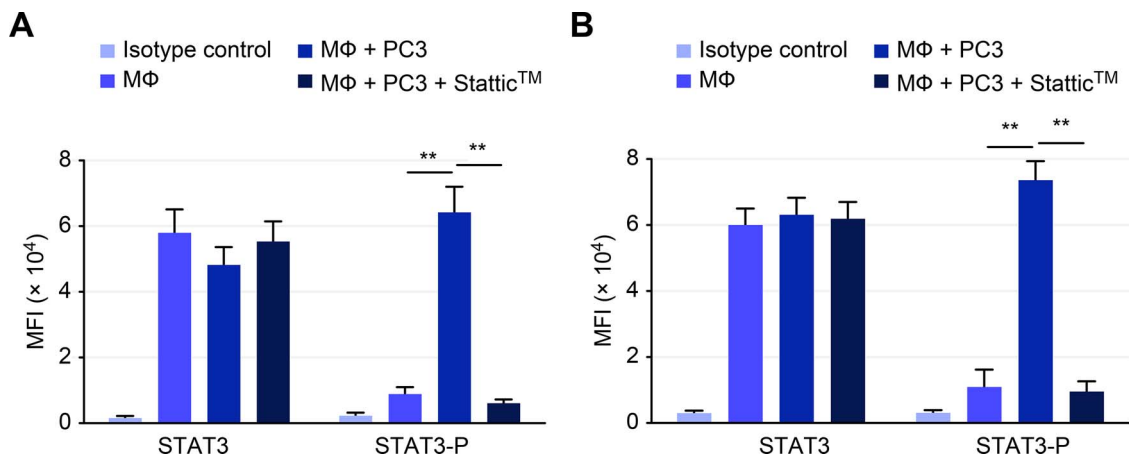


Fig. 1. A) STAT3 and STAT3-P expression in macrophages treated for 48 h with either 30% v/v PC3 culture supernatant (MØ + sPC3), macrophages treated for 1 h with 50 µM Stattic™ and then for 48 h with 30% PC3 culture supernatant (MØ + sPC3 + Stattic™), and macrophages under baseline conditions (MØ) were determined using flow cytometry. B) In the same experimental conditions the supernatant of tumor cells was changed for 1×10^6 PC3 cells using transwell plaques. Mann-Whitney *U* tests were performed to compare means between groups. **p* < 0.05; ns = Not significant. Data are shown as the mean ± Standard Deviations (SD) of three independent experiments performed in triplicate. STAT3: signal transducer and activator of transcription 3; P: the phosphorylated state.

performed using GraphPad Prism statistical software ver. 6.0. Continuous variables were compared using the Mann-Whitney *U* test, and *p* < 0.05 was considered to be statistically significant.

Ethical considerations: All volunteers signed an informed consent agreement, and the protocol was authorized by the Scientific and Ethical Committee of the CIBO-IMSS Institution (R-2015-1305-2).

3. Results

3.1. Phosphorylation of STAT3 in human peripheral blood macrophages by incubation with PC3 tumor-cell supernatant and its inhibition by stattic™

First, we determined whether supernatant from prostatic-tumor cell cultures modified the expression of transcription factor STAT3. Fig. 1A shows that, when macrophages were incubated with 30% v/v tumor-cell supernatant, the MFI of endogenous STAT3 practically did not change, this was due to that the values in STAT3 expression were in all groups were between 48,000 and 58,000 of MFI and similar to those of the untreated control group regardless of whether cells were treated with PC3 tumor-cell supernatant alone or in the presence of Stattic™. However, active phosphorylated STAT3 in the untreated control macrophages were observed with an MFI = 8876. In the presence of PC3 tumor-cell supernatant, an increase of phosphorylated STAT3 was observed and researched, with a $\Delta\%$ = 2330%; this group returned to the baseline value when the cells were incubated with the Stattic™ inhibitor. Practically the same behavior was observed on employing a Transwell method (Fig. 1B) to incubate macrophages with PC3 cells than by incubating the macrophages exclusively with the supernatant of the tumor line. Taken together, these results confirm that the secretion products of prostate- cancer cells activate STAT3 in macrophages.

3.2. PC3 tumor cancer-cell supernatant induces the M2 phenotype in peripheral blood macrophages and the participation of STAT3

The objective of the present experiment was achieved by monitoring changes in the macrophage phenotype induced by PC3-cell supernatant and by the inhibition of STAT3 with Stattic™ using flow cytometry.

The results depicted in Fig. 2A indicate that, as expected, the traditional M1 macrophage marker CD14 was present in 100% of cells from the untreated control group or the M1 phenotype-induced group, while TNFR1 was present in 89.1% and 95%, respectively, supporting the validity of the experiment.

In contrast, when the cells were incubated with either IL-4, IL-10, or PC3 tumor-cell supernatant, the M1 markers practically disappeared in untreated control and M1-induced groups. In this same Fig. 1A, it was interesting to observe that, in macrophages incubated with PC3 supernatant, the presence of the STAT3 inhibitor recovered 100% of the expression of CD11a, CD86, and TNFR1 values, which was strictly comparable to untreated or M1- induced cells. However, only 60% of CD14 expression was reached in comparison to that of the respective control groups.

We also studied the changes in expression in M2 phenotype markers CD163 and CD206 (Fig. 2B). In the control, untreated macrophages, as well as in the induced M1 macrophage phenotype, both markers were weakly expressed. However, the addition of PC3 tumor-cell supernatant to cultures induced a nearly 100% increase in CD163- or CD206-positive cells, in comparison with the control group, and pre-treatment with the STAT3 inhibitor reversed this change to values similar to those of the untreated or the M1-induced group.

Because the density of receptor expression plays an important role in the activity of cells, in the same experiment, we also determined the expression density of the studied receptors by measuring the MFI.

Fig. 3 demonstrates that the different treatments modified not only the percentage of positive cells, but also the number of receptors in the cells, in the same sense of the percentage. In general, the number of M1- and M2-associated receptors increased when the cells were incubated with either the M1 or M2 interleukin profile in comparison with the respective control groups. The addition of the STAT3 inhibitor did not modify M1 or M2 surface markers in comparison with untreated control cells, and in the group of macrophages incubated with the PC3 supernatant, an increase of M2 markers near the corresponding values of M2-induced macrophages with IL-10 + IL4 were observed. Finally, in the group incubated with PC3 supernatant + Stattic™, it is clearly perceived that M1 markers are very close to those of the corresponding untreated control group and M2 markers practically disappear. Together, these results strongly suggest that the PC3 supernatant not only induces a higher percentage of cells with an M2 phenotype, but that it also importantly modifies the membrane density of these macrophage receptors.

Together, these experiments strongly suggest the importance of the microenvironment induced by the supernatant from PC3 tumor cells and the direct participation of transcription factor STAT3 in the induction of the M2 profile, which was verified by Stattic™ treatment, confirming our hypothesis.

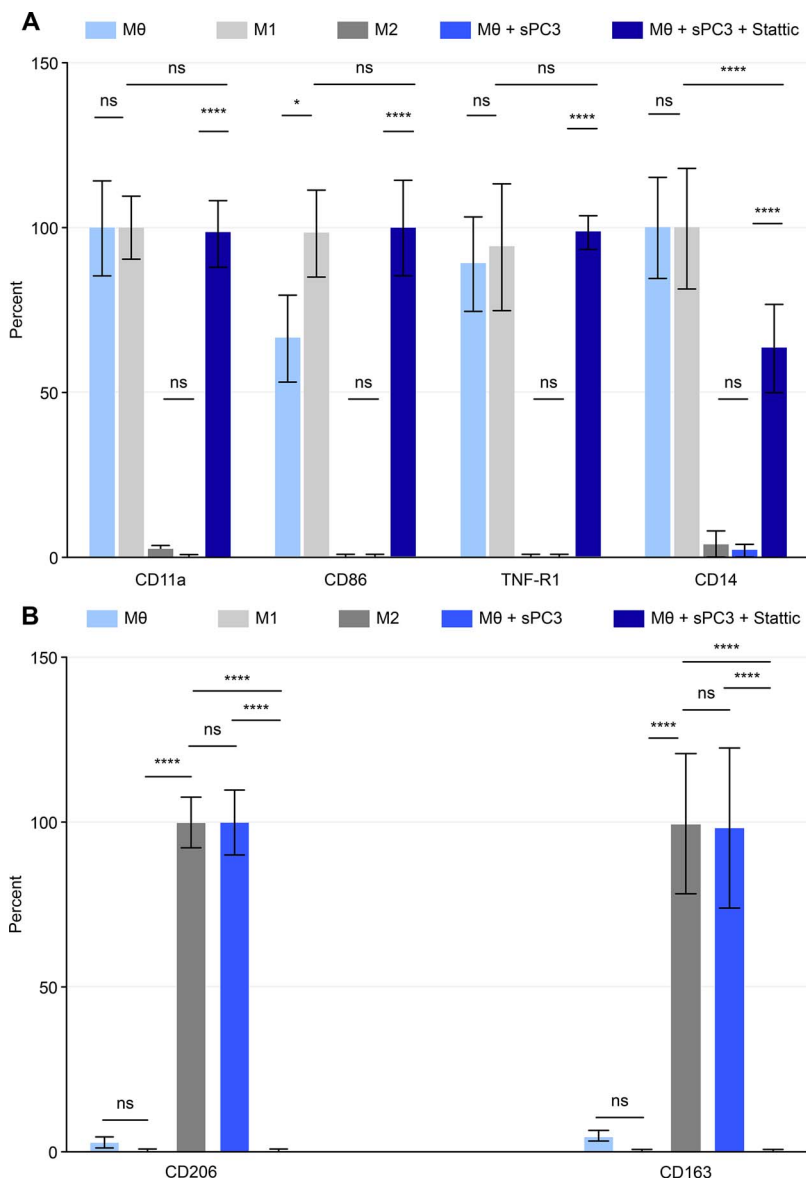


Fig. 2. Expression of membrane receptors related to the M1(A) and M2 (B) macrophage phenotypes. Membrane receptors are presented as percent (%). The M0 macrophage group (macrophage group without any treatment), M1 macrophage group (incubated with 20 ng/mL IFN- γ + 100 ng/mL LPS), and M2 macrophage group (incubated with 20 ng/mL of both IL-10 and IL-4) represent the control, M1 and M2 groups, respectively. M0 + sPC3 represents the experimental group (M0 + 30% v/v prostate cancer PC3 cell supernatant) and M0 + sPC3 + Statistic™ represents the other experimental group (50 μ M Statistic™). Mann-Whitney *U* tests were performed to compare means between groups. **p* < 0.05, and *****p* < 0.001. ns = not significant. Data are shown as the mean \pm Standard Deviations (SD) of three independent experiments performed in triplicate. TNFRI: tumor necrosis factor receptor-I.

3.3. Incubation with the supernatant of PC3 prostate-cancer cells modify the pattern of secretion of interleukins and of the growth factors of peripheral blood macrophages

Because certain growth factors can influence macrophage activities, we decided to quantify the concentrations of FGF, TNF- α , G-CSF, GM-CSF, M-CSF, PDGF-A, and TGF- β in the supernatant of the macrophage

cultures of all of the groups under the same experimental conditions. When the macrophages were cultured with PC3-cell supernatant, the concentration of all growth factors was between 15 and 97 pg/mL, as presented in Fig. 4, while in the growth factors measured in the macrophage control group without any stimulus, secretion of growth factors was minimal (3–5 pg/mL). Then, the control macrophages were incubated exclusively with Statistic™ to determine whether the STAT3

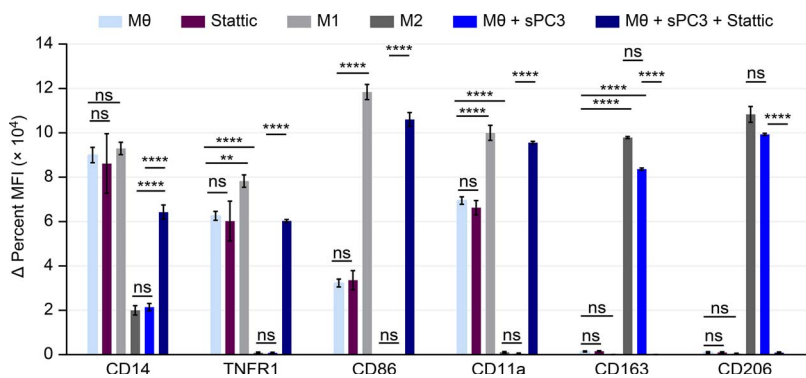


Fig. 3. Delta percent (Δ %) of the MFI (mean fluorescence intensity) of the expression of membrane receptors and transcription factors related to macrophages cultivated with 30% PC3 prostate cancer cell line supernatant (M0 + sPC3) and macrophages cultivated for one hour with 50 μ M Statistic™ and then with 30% PC3 prostate cancer cell line supernatant (M0 + sPC3 + Statistic™). The control groups were M0 macrophages (macrophage group without any treatment), M1 macrophages (treated with 20 ng/mL of IFN- γ + 100 ng/mL LPS), and M2 macrophages (treated with 20 ng/mL IL-10 + IL-4 of both). Mann-Whitney *U* tests were performed to compare the means of experimental groups and M0 with M0 + sPC3. ***p* < 0.05. *****p* < 0.001. ns = not significant. Data are shown as the mean \pm Standard Deviations (SD) of three independent experiments performed in triplicate. STAT3: signal transducer and activator of transcription 3; p: the phosphorylated state.

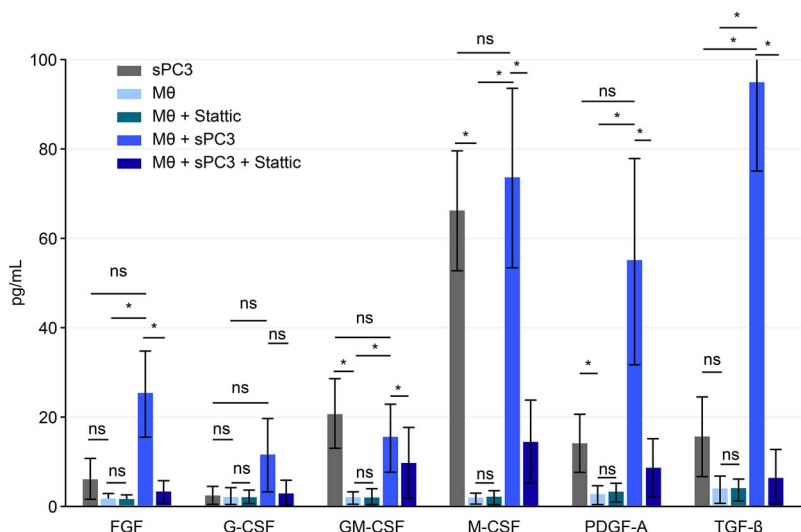


Fig. 4. Profile of growth factors secreted by human macrophages. Expression in macrophages treated 48 h with 30% v/v PC3 cell culture supernatant (M0 + sPC3) and macrophages treated for one hour with 50 μM Stattic™ and then for 48 h with 30% v/v of PC3 cell culture supernatant (M0 + sPC3 + Stattic™). The control groups were M0 macrophages (macrophage group without any treatment) and macrophages treated with PC3 cell supernatant (30% v/v). Mann-Whitney U tests were performed to compare means between groups. *p < 0.05. Data are shown as the mean ± Standard Deviations (SD) of three independent experiments performed in triplicate. TGF-β: transforming growth factor beta; PDGF-A: platelet-derived growth factor-A; M-CSF: macrophage colony-stimulating factor; GM-CSF: macrophage-granulocyte colony-stimulating factor; G-CSF: granulocyte colony-stimulating factor; FGF: fibroblast growth factor.

inhibitor induces modification per se, and we observed that the levels of all of the growth factors were comparable to those secreted by macrophages incubated exclusively in culture medium without any stimulus. Remarkably, when the cells were incubated with PC3 tumor-cell supernatant, in all cases, the secretion of all growth factors was strongly stimulated, with a minimal stimulation of Δ % = 233% for G-CSF and a maximal stimulation for TGF-β with a Δ % = 1300%.

In agreement with prior experiments, in all cases, we observed that with the addition of the STAT3 inhibitor (Stattic™), concentration of the growth factors was nearly that of the values of the macrophage cultures without stimulus, with the exception of M-CSF which is approximately five times higher.

We also determined the cytokine profile found in the supernatant of PC3 cancer cells. Fig. 5 demonstrates that levels in the PC3-cell supernatant of cytokines IL-10 and IL-4 (approximately 20 pg/mL) and, to a lesser degree, of IL-6 (15 pg/mL), are higher in comparison with those of the supernatant from untreated control cells. In this experiment, we also confirmed that the presence of the STAT3 inhibitor in macrophage culture does not alter the secretion of the different cytokines. Notably, in the untreated control group, INF-γ and TNF-α cytokines, considered as M1 profile, exhibit similar behavior to M1, with values of nearly

20 pg/mL. As predicted in the literature, in our present results, we observed a very important increase in the secretion of IL-10 (Δ % = 690%) and IL-4 (Δ % = 712), but, in contrast, very weak expression of IFN-γ was measured in the culture of macrophages incubated with the PC3-cell supernatant. We also observed lower levels of IL-6 secretion. As in the other experiments, with the addition of Stattic™, the STAT3 inhibitor, a very important diminution of IL-10 and IL-4 levels was observed, with values near those of the untreated control group, and concentrations of TNF-α and IFN-γ returned to the same level as those found in the supernatant from macrophages cultured without stimulation.

3.4. Participation of IL-4, IL-6, and IL-10 in the M2 immunophenotype induced by the PC3 tumor-cell supernatant

Because we observed that STAT3 phosphorylation plays a very important role in the transformation of the M1 into the M2 immunophenotype and that this is related with interleukins, in order to study its participation in our observations, we inhibited interleukins activity utilizing antibodies and, confirming the previous experiments, Fig. 6 reveals that the presence of PC3 supernatants induces higher

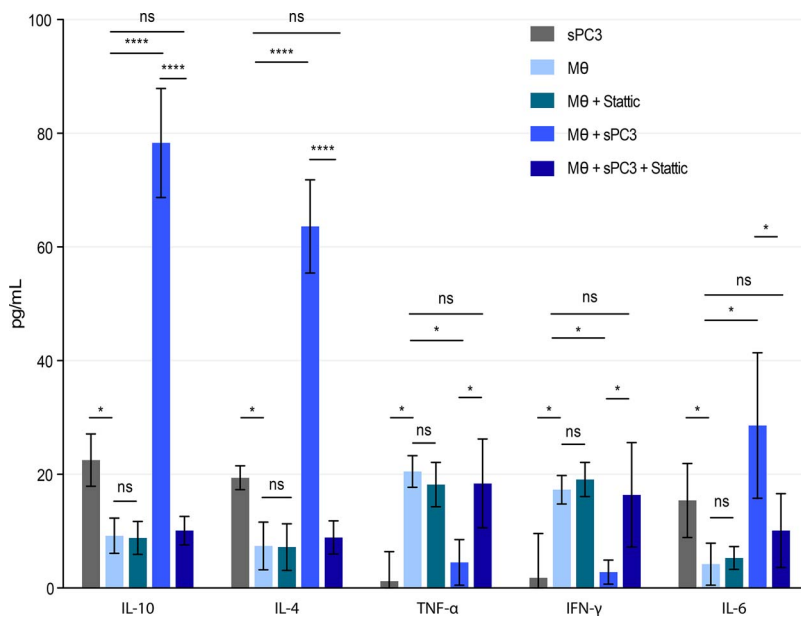


Fig. 5. Profile of cytokines secreted by human macrophages. Cytokine expression in macrophages cultured for 48 h with 30% v/v PC3 culture supernatant (M0 + sPC3), and in the experimental group, macrophages treated for one hour with 50 μM Stattic™ and then for 48 h with 30% PC3 cell culture supernatant (M0 + sPC3 + Stattic™). The control groups were M0 macrophages (macrophage group without any treatment) and macrophages treated with PC3 cell supernatant (30% v/v). Mann-Whitney U tests were performed to compare means between groups. *p < 0.05, and ****p < 0.001. ns = not significant. Data are shown as the mean ± Standard Deviations (SD) of three independent experiments performed in triplicate. TNF-α: tumor necrosis factor alpha; IFN-γ: interferon gamma.

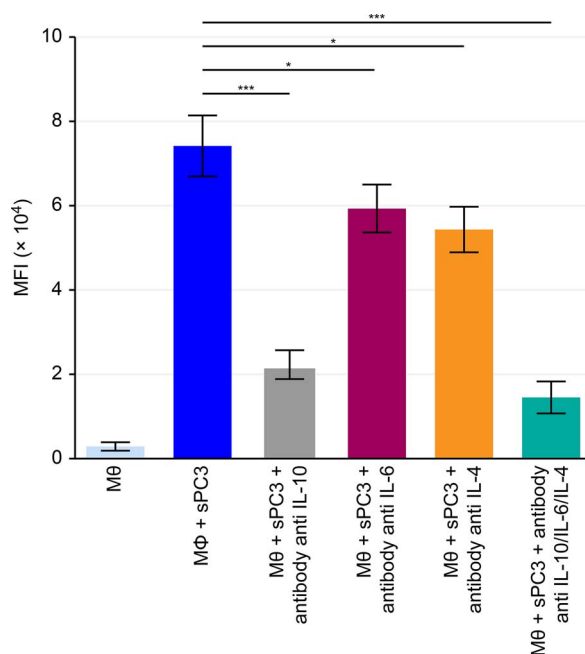


Fig. 6. Peripheral blood macrophages incubated with supernatant of PC3, effects of interleukin inhibition. As above experiments, the macrophages were incubated with 30% v/v during 48 h either in the presence or not of IL-10 (50 µg/mL), IL-6 (40 µg/mL), IL-4 (50 µg/mL) or its combination. To end of incubation STAT3 phosphorylation was determined by flow cytometry. The results are expressed as Median fluorescence intensity (MFI) and represent the mean ± Standard Deviation of three independent experiments carried out in triplicate, Mann-Whitney *U* tests were performed to compare means between groups. *** *p* < 0.001; **p* < 0.05; ns = Not significant. P = phosphorylated state.

phosphorylation of STAT3 (MFI = 74,198) with a $\Delta\%$ = 735%, in comparison with the untreated control group (MFI = 8876; *p* < 0.05). The macrophage cultures effected in the presence of PC3 supernatant + IL-6 or IL-4 antibodies demonstrate similar values compared among themselves, but values lower than those of the macrophage group cultured in the presence of the PC3 supernatant without antibodies

($\Delta\%$ = -22.4% and $\Delta\%$ = -31.0%, respectively). In contrast, very significant inhibition of STAT3 phosphorylation was induced by IL-10 inhibition in the PC3 supernatant-cultured group

(MFI = 22,314) with a $\Delta\%$ = -70.16% was observed as compared with the cultures of macrophages with only with PC3 supernatant added (*p* < 0.05). Additionally, in the culture of macrophages added to the PC3 supernatant + antibodies against IL-10, IL-6, and IL-4, there were practically no differences between this group and the group treated with antibodies against IL-10, suggesting a very important participation of this interleukin in our observations.

3.5. In-vitro PC3 supernatant inhibits the cytotoxicity activity of macrophages against PC3 tumor cells and its recovery by STAT 3 inhibition

The antitumor activity of macrophages is very important and comprises a constitutive and powerful weapon of natural immunity. In this experiment, we studied how PC3 cancer-cell supernatant modified cytotoxic activity in M1 and M2 macrophages. The results illustrated in Fig. 7 demonstrate that the cytotoxicity of control macrophages without any stimulus was 59.5 ± 6.7%, 44.2 ± 5.5%, and 24.7 ± 6.9% for the E:T ratios of 40:1, 20:1, and 10:1, respectively, and that the values are comparable to macrophages incubated exclusively with Statix™. The addition of IFN-γ + LPS induced an important increase in lactic acid release from target cells (71.2 ± 4.8%; E:T, 40:1). In contrast, an important attenuation was observed with macrophages incubated with IL-10 and IL-4 (14.1 ± 6.8%; E:T, 40:1), which was similar to the

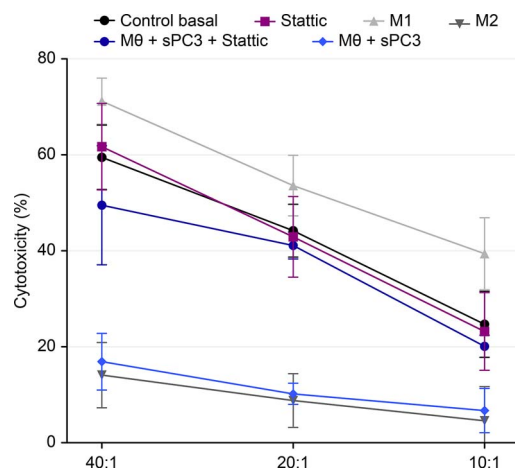


Fig. 7. Cytotoxicity measured by % lactic acid release associated with macrophages cultured with 30% v/v of PC3 prostate cancer cell supernatant (MØ + sPC3); other experimental group macrophages were cultured for one hour with 50 µM Statix™ and then for 48 h with 30% v/v PC3 cell-culture supernatant (MØ + sPC3 + Statix™). The control groups were MØ macrophages (control group), macrophages treated for one hour with 50 µM Statix™, M1 macrophages (treated with 20 ng/mL of IFN-γ + 100 ng/mL LPS) and M2 macrophages (treated with 20 ng/mL IL-10 + IL-4 of both). Data are shown as the mean ± Standard Deviations (SD) of three independent experiments performed in triplicate.

cytotoxicity percentage exhibited in the culture group treated with the PC3-cell supernatant (16.9 ± 5.9%; E:T, 40:1). However, inhibition of STAT3 induced high recovery of the percentage of macrophage cytotoxicity (49.5 ± 12.4%; E:T, 40:1), which was not significantly different from that of the untreated control group.

3.6. Inhibition of STAT3 recovers the nitric oxide secretion by peripheral blood macrophages incubated with PC3 tumor- cell supernatant

Macrophages were incubated under the same experimental conditions, and the secretion of nitric oxide, which is an important marker of macrophage activation, was measured using the Griess technique. Fig. 8 shows that nitric oxide secretion in the macrophage culture without stimulus reached 50.2 ± 7.8 µmol/L. Obviously, when the cells were stimulated with IFN-γ plus LPS, this production was significantly increased (72.3 ± 9.8 µmol/L). In addition, similar to the preceding experiments, when the cells were incubated with PC3-cell supernatant, we observed the same concentration of nitric oxide (29.1 ± 8.3 µmol/L) as that with macrophages incubated with IL-4 and IL-10. However, in the group incubated with PC3- cell supernatant + Statix™, an important recovery of nitric oxide secretion near the level of that of the untreated group was detected. These results, along with those of the preceding experiments, confirm that the supernatant of PC3 cells inhibits the function of macrophages, but that this can be recovered by inhibiting transcription factor STAT3.

3.7. Disclosure statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

4. Discussion

The work allows us to affirm that the soluble derivative products of the PC3 prostate-cancer cells induce the phosphorylation of STAT3 with the consequent transformation of phenotype macrophages M1 into M2, confirmed by the supernatant and Transwell experiments, and it is very likely that *in-vivo* tumor peripheral macrophages exhibit a similar behavior. However, it is important to consider that in a

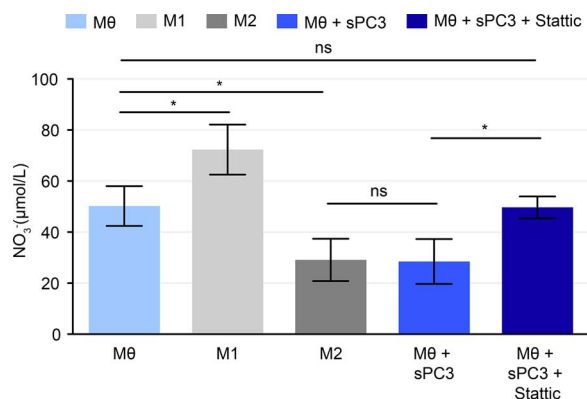


Fig. 8. Nitric Oxide (NO₃⁻) secretion by macrophages cultivated with 30% PC3 prostate cancer cell supernatant (M0 + sPC3) and macrophages treated with 50 μM Static™ and then cultured with 30% v/v of PC3 prostate cancer cell supernatant (M0 + sPC3 + Static™). The control groups were M0 macrophages (control group), M1 macrophages (treated with 20 ng/mL of IFN-γ + 100 ng/mL LPS) and M2 macrophages (treated with 20 ng/mL IL-10 + IL-4 of both). Mann-Whitney *U* tests were performed to compare means between experimental groups, M0 with M0 + sPC3 and M0 with M0 + sPC3 + Static™. **p* < 0.05. ns = not significant. Data are shown as the mean ± Standard Deviations (SD) of three independent experiments performed in triplicate. STAT3: signal transducer and activator of transcription 3.

microenvironment, the behavior of macrophage direct cell-to-cell contact may not be strictly comparable.

The M2 macrophage phenotype, which is known to be favorable for cancer development, promotes tissue remodeling and tumor progression and possesses immunoregulatory functions [21–24]. Thus, it plays an important role in tumor development.

The M2 phenotype observed when cells were incubated with PC3 tumor-cell supernatant or co-cultured with macrophages exhibited an increased percentage of CD206- and CD163-positive cells (M2 phenotype markers), as well as increased CD206 and CD163 density according to flow cytometry [25], and diminished the expression of CD11a, CD86, and TNFR1 (M1 phenotype markers) [26]. Therefore, the PC3 tumor-cell supernatant achieved the same effect as cultures with IL-4 and IL-10, which are known M2-phenotype inducers [27].

However, the profile of membrane proteins was comparable with an M2-like phenotype, and the functional and anti-tumor activity of the macrophages was similar when the macrophages were cultured with either PC3 tumor prostate cancer-cell supernatant or IL-4/IL-10. The results obtained in both groups were comparable. Compared with the untreated control groups, cytotoxicity capacity was only 30% in both groups, and the release of nitric oxide diminished to one half of that observed in the untreated control group.

The main finding of this work is the phosphorylation of transcription factor STAT3. This transcription factor is constitutively active in many types of tumors, including prostate-cancer tumors, and reports have indicated that STAT3 is phosphorylated in 71% or 82% [28] of primary prostate-tumor specimens and activated in diverse cancer-cell lines [29–31]. Our observations indicate that phosphorylation of STAT3 is induced by the PC3 supernatant in a manner similar to that in cells incubated with IL-4/IL-10. Under our experimental conditions, when phosphorylation of this transcription factor is impeded by its specific inhibitor Static™, the PC3 tumor-cell supernatant does not induce STAT3 phosphorylation, macrophages are converted into an M1 phenotype, and nitric oxide secretion and anti-tumor activity are recovered. In one report, STAT3 in macrophages was blocked with an STAT3-specific oligonucleotide decoy in a rat model of breast cancer, and recovery of the antitumor activity of the macrophages was observed [32], similar to our results.

Our results agree with other reports in the literature; thus, other researchers have triggered STAT3 phosphorylation in macrophages obtained from volunteer donors treated with supernatants from a panel of human tumor cells [33]. Also, our results are in agreement with the

observations of Han et al. [34], who studied Static™ in prostate-cancer cells.

In 1992, Stein and Gordon demonstrated that IL-4 could induce macrophages toward an M2 phenotype [35] and that IL-4 regulates the expression of a CD206 (macrophages mannose receptor) [36]. Another interleukin that is highly expressed in this group of macrophages was IL-6, and the latter has been demonstrated to induce STAT3 activation [39]. Xu et al. [40] found amounts of IL-6 in the supernatant of the PC3 cancer-cell line that was nearly equal to those found in this study [37,38]. IL-10 is known as a powerful immunosuppressor, and over-expression of IL-10 is associated with a worse prognosis in solid tumors [39] and induces STAT3 phosphorylation [40]. Also, based on inhibition experiments of interleukins with antibodies, the change-of-phenotype of macrophages of M1 into M2, as well as the phosphorylation status of STAT3, allow us, under our experimental conditions, to state that IL-10 plays a central role and these observations are in agreement with previous works [41].

Taken together, the interleukin results strongly suggest that the elevation of IL-4, IL-6, and IL-10 were dependent on STAT3 phosphorylation.

M-CSF and GM-CSF are considered the first-stage markers of macrophage differentiation and can induce macrophages toward an M2 or an M1 phenotype, respectively [21,43,44]. Our results demonstrated that the growth factor with highest value in the PC3 cancer cell-line supernatant was M-CSF, and these results are in agreement with those of Savarese et al. [45], who found M-CSF in the same prostate cancer cell-line supernatant at a concentration of > 70 pg/mL, values near those of our observations. Levels of growth factors G-CSF, with values of < 10 pg/mL, and GM-CSF, with levels of > 25 pg/mL, were also in agreement with this previous report [45].

The growth factor TGF-β exhibited an even higher increase (*p* < 0.05). TGF-β is a well-known immune suppressor in both innate and adaptive immunity. Regulation of inducible Nitric Oxide Synthase (iNOS) at the transcriptional level and of TNF-α at the post-transcriptional level [44] explains why nitric oxide and TNF-α were nearly undetectable in the PC3 incubation group. An elevated concentration of TGF-β has also been demonstrated in a prostate cancer-cell line and is associated with tumor progression and metastasis [47–49]. The M2-like phenotype is an important immunological cell that produces and secretes this growth factor. Other growth factors, such as FGF, M-CSF, and PDGF-A, exhibited an increased concentration, but the increase was not significant (*p* > 0.05), suggesting little participation in our observations. On the other hand, the concentration of GM-CSF diminished in PC3-cultured cells, which is likely because the population of macrophages at 48 h of incubation exhibited an M2-like phenotype.

Notably, when macrophages were incubated with PC3 supernatant plus Static™, all of these growth factors were downregulated, and the values were not significantly different from those of the untreated control group, with the exception of M-CSF. Instead, the levels of the M1 profile cytokines TNF-α, and IFN-γ, which had lowest values in the group of macrophages cultured with 30% prostate cancer cell-line supernatant, returned to levels comparable to those of the control group (macrophages without stimulus) when the macrophages were treated with the STAT3 inhibitor Static™. This indicates that inhibition of STAT3 phosphorylation by Static™ also reverted macrophages to an M1 phenotype, in agreement with recovery of the anti-tumor activity of the macrophages.

5. Conclusion

The results obtained in our study reveal that the secretion profile of PC3 prostate-cancer cells induces a phenotypic change in macrophages from M1 into M2, and this phenomenon is related to an increase of IL-10 and phosphorylation of transcription factor STAT3. These results contribute to our understanding of prostate-tumor behavior and may be useful for designing new treatments.

Acknowledgments

This work was supported by FIS-IMSS-PROT-G15-1437, and we thank Net INMUNICANEI (Net of Cancer Immunology and Infectious Diseases)-CONACYT 253053 and José Francisco Muñoz-Valle, Ph.D.

References

- [1] R.L. Siegel, K.D. Miller, A. Jemal, Cancer statistics, CA. Cancer J. Clin. 66 (2016) 7–30.
- [2] P.A. McArdle, K. Canna, D.C. McMillan, A.M. McNicol, R. Campbell, M.A. Underwood, The relationship between T-lymphocyte subset infiltration and survival in patients with prostate cancer, Br. J. Cancer 91 (2004) 541–543.
- [3] S. Vesalainen, P. Lipponen, M. Talja, K. Syrjänen, Histological grade, perineural infiltration, tumour-infiltrating lymphocytes and apoptosis as determinants of long-term prognosis in prostatic adenocarcinoma, Eur. J. Cancer 30A (1994) 1797–1803.
- [4] M.R. Hussein, M. AL-Assiri, A.O. Musalam, Phenotypic characterization of the infiltrating immune cells in normal prostate, benign nodular prostatic hyperplasia and prostatic adenocarcinoma, Exp. Mol. Pathol. 86 (2009) 108–113.
- [5] K.S. Sfanos, A.M. de Marzo, Prostate cancer and inflammation: the evidence, Histopathology 60 (2012) 199–215.
- [6] T. Chanmee, P. Ontong, K. Konno, N. Itano, Tumor-associated macrophages as major players in the tumor microenvironment, Cancers 6 (2014) 1670–1690.
- [7] M. Lanciotti, L. Masieri, M.R. Raspollini, A. Minervini, A. Mari, G. Comito, E. Giannoni, M. Carini, P. Chiarugi, S. Serni, The role of M1 and M2 macrophages in prostate cancer in relation to extracapsular tumor extension and biochemical recurrence after radical prostatectomy, BioMed. Res. Int. 2014OJO (2014) 486798.
- [8] N. Nonomura, H. Takayama, M. Nakayama, Y. Nakai, A. Kawashima, M. Mukai, A. Nagahara, K. Aozasa, A. Tsujimura, Infiltration of tumor-associated macrophages in prostate biopsy specimens is predictive of disease progression after hormonal therapy for prostate cancer, BJU Int. 107 (2000) 1918–1922.
- [9] S. Shimura, G. Yang, S. Ebara, T.M. Wheeler, A. Frolov, T.C. Thompson, Reduced infiltration of tumor-associated macrophages in human prostate cancer: association with cancer progression, Cancer Res. 60 (2000) 5857–5861.
- [10] A. Sica, T. Schioppa, A. Mantovani, P. Allavena, Tumour-associated macrophages are a distinct M2 polarised population promoting tumour progression: potential targets of anti-cancer therapy, Eur. J. Cancer 42 (2006) 717–727.
- [11] P.G. Través, A. Luque, S. Hortelano, Macrophages, inflammation, and tumor suppressors: ARF, a new player in the game, Mediators Inflamm. 2012 (2012) 568783.
- [12] F. Sheikh, H. Dickensheets, J. Pedras-Vasconcelos, T. Ramalingam, L. Helming, S. Gordon, R.P. Donnelly, The interleukin-13 receptor- α 1 chain is essential for induction of the alternative macrophage activation pathway by IL-13 but not IL-4, J. Innate Immun. 7 (2015) 494–505.
- [13] T.A. Wynn, K.M. Vannella, Macrophages in tissue repair, regeneration, and fibrosis, Immunity 44 (2016) 450–462.
- [14] F.O. Martínez, S. Gordon, M. Locati, A. Mantovani, Transcriptional profiling of the human monocyte-to-macrophage differentiation and polarization: new molecules and patterns of gene expression, J. Immunol. 177 (2006) 7303–7311.
- [15] D.J. Nikolic-Paterson, S. Wang, H.Y. Lan, Macrophages promote renal fibrosis through direct and indirect mechanisms, Kidney Int. Supply 4 (2011) 34–38.
- [16] L.Y. Fang, K. Izumi, K.P. Lai, L. Liang, L. Li, H. Miyamoto, W.J. Lin, C. Chang, infiltrating macrophages promote prostate tumorigenesis via modulating androgen receptor-mediated CCL4-STAT3 signaling, Cancer Res. 73 (2013) 5633–5646.
- [17] W. Luo, B. Yin, Q.-Y. Qin, J.-M. Lu, S.-Y. Qin, H.-X. Jiang, Interleukin-22 promotes macrophages M2 polarization via STAT3 pathway, Int. J. Clin. Exp. Med. 9 (2016) 19574–19580.
- [18] J.L. Bishop, D. Thaper, A. Zoubeidi, The multifaceted roles of STAT3 signaling in the progression of prostate cancer, Cancers 6 (2014) 829–859.
- [19] J. Turkson, R. Jove, STAT proteins: novel molecular targets for cancer drug discovery, Oncogene 19 (2000) 6613–6626.
- [20] J. Schust, B. Sperl, A. Hollis, T.U. Mayer, T. Berg, Stattic: a small-molecule inhibitor of STAT3 activation and dimerization, Chem. Biol. 13 (2006) 1235–1242.
- [21] S. Gordon, F.O. Martínez, Alternative activation of macrophages: mechanism and functions, Immunity 32 (2010) 593–604.
- [22] S.K. Biswas, A. Mantovani, Macrophage plasticity and interaction with lymphocyte subsets: cancer as a paradigm, Nat. Immunol. 11 (2010) 889–896.
- [23] A. Sica, V. Bronte, Altered macrophage differentiation and immune dysfunction in tumor development, J. Clin. Invest. 117 (2007) 1155–1166.
- [24] A. Mantovani, S. Sozzani, M. Locati, P. Allavena, A. Sica, Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes, Trends Immunol. 23 (2002) 549–555.
- [25] C. Buechler, M. Ritter, E. Orsó, T. Langmann, J. Klucken, G. Schmitz, Regulation of scavenger receptor CD163 expression in human monocytes and macrophages by pro- and anti-inflammatory stimuli, J. Leukoc. Biol. 67 (2000) 97–103.
- [26] L.B. Boyette, C. Macedo, K. Hadi, B.D. Elinoff, J.T. Walters, B. Ramaswami, G. Chalasani, J.M. Taboas, F.G. Lakkis, D.M. Metes, Phenotype, function, and differentiation potential of human monocyte subsets, PLoS One 12 (2017) e0176460.
- [27] E.B. Lurier, D. Dalton, W. Dampier, P. Raman, S. Nassiri, N. Ferraro, R. Rajagopalan, M. Sarmady, K.L. Spiller, Transcriptome analysis of IL-10-stimulated (M2c) macrophages by next-generation sequencing, Immunobiology 7 (2017) 847–856.
- [28] L.B. Mora, R. Buettner, J. Seigne, J. Díaz, N. Ahmad, R. García, T. Bowman, R. Falcone, R. Fairclough, A. Cantor, C. Muro-Cacho, S. Livingston, J. Karras, et al., Constitutive activation of Stat3 signaling induces apoptosis of in human tumors provides novel molecular targets for therapeutic intervention, Clin. Cancer 8 (2002) 945–954.
- [29] M. Kortylewski, R. Jove, H. Yu, Targeting STAT3 affects melanoma on multiple fronts, Cancer Metastasis Rev. 24 (2005) 315–327.
- [30] T.S. Lin, S. Mahajan, D.A. Frank, STAT signaling in the pathogenesis and treatment of leukemias, Oncogene 19 (2000) 2496–2504.
- [31] P.J. Coffer, L. Koenderman, R.P. de Groot, The role of STATs in myeloid differentiation and leukemia, Oncogene 19 (2000) 2511–2522.
- [32] Z. Sun, Z. Yao, S. Liu, H. Tang, X. Yan, An oligonucleotide decoy for Stat3 activates the immune response of macrophages to breast cancer, Immunobiology 211 (2006) 199–209.
- [33] P. Vlaicu, P. Mertins, T. Mayr, P. Widschwendter, B. Ataseven, B. Högel, W. Eiermann, P. Knyazev, A. Ullrich, Monocytes/macrophages support mammary tumor invasivity by co-secreting lineage-specific EGFR ligands and a STAT3 activator, BMC Cancer 13 (2013) 197.
- [34] Z. Han, X. Wang, L. Ma, L. Chen, M. Xiao, L. Huang, Y. Cao, J. Bai, D. Ma, J. Zhou, Z. Hong, Inhibition of STAT3 signaling targets both tumor-initiating and differentiated cell populations in prostate cancer, Oncotarget 5 (2014) 8416–8428.
- [35] M. Stein, S. Keshav, N. Harris, S. Gordon, Interleukin 4 potentially enhances murine macrophage mannose receptor activity: a marker of alternative immunologic macrophage activation, J. Exp. Med. 176 (1992) 287–292.
- [36] N.B. Hao, M.H. Lü, Y.H. Fan, Y.L. Cao, Z.R. Zhang, S.M. Yang, Macrophages in tumor microenvironments and the progression of tumors, Clin. Dev. Immunol. 2012 (2012) 948098.
- [37] H. Sumimoto, F. Imabayashi, T. Iwata, Y. Kawakami, The BRAF-MAPK signaling pathway is essential for cancer-immune evasion in human melanoma cells, J. Exp. Med. 203 (2006) 1651–1656.
- [38] K.W. Moore, R.W. de Waal Malefyt, R.L. Coffman, A. O'Garra, Interleukin-10 and the interleukin-10 receptor, Annu. Rev. Immunol. 19 (2001) 683–765.
- [39] S. Zhao, D. Wu, P. Wu, Z. Wang, J. Huang, Serum IL-10 predicts worse outcome in cancer patients: a meta-analysis, PLoS One 10 (2015) e0139598.
- [40] H. Yasukawa, M. Ohishi, H. Mori, M. Murakami, T. Chinen, D. Aki, T. Hanada, K. Takeda, S. Akira, M. Hoshijima, T. Hirano, K.R. Chien, A. Yoshimura, IL-6 induces an anti-inflammatory response in the absence of SOCS3 in macrophages, Nat. Immunol. 4 (2003) 551–556.
- [41] M. Zhou, H. Yang, R.M. Learned, H. Tian, L. Ling, Non-cell-autonomous activation of IL-6/STAT3 signaling mediates FGF19-driven hepatocarcinogenesis, Nat. Commun. 8 (2017) 15433.
- [42] F.O. Martínez, S. Gordon, The M1 and M2 paradigm of macrophage activation: time for reassessment, F100 Prime Rep. 6 (2014) 13.
- [43] E. Van Overmeire, B. Stijlemans, F. Heymann, J. Keirsse, Y. Morias, Y. Elkrin, L. Brys, C. Abels, Q. Lahmar, C. Ergen, L. Vereecke, F. Tacke, P. De Baetselier, J.A. Van Ginderachter, D. Laoui, M.-C.S.F and GM-CSF receptor signaling differentially regulate monocyte maturation and macrophage polarization in the tumor microenvironment, Cancer Res. 76 (2016) 35–42.
- [44] D.M. Savarese, H. Valinski, P. Quesenberry, T. Savarese, Expression and function of colony-stimulating factors and their receptors in human prostate carcinoma cell lines, Prostate 34 (1998) 80–91.
- [45] P. Wikström, P. Stattin, I. Franck-Lissbrant, J.E. Damber, A. Bergh, Transforming growth factor beta1 is associated with angiogenesis, metastasis, and poor clinical outcome in prostate cancer, Prostate 37 (1998) 19–29.
- [46] J.A. Eastham, L.D. Truong, E. Rogers, M. Kattan, K.C. Flanders, P.T. Scardino, T.C. Thompson, Transforming growth factor-1: comparative immunohistochemical localization in human primary and metastatic prostate cancer, Lab. Invest. 3 (1995) 628–635.
- [47] L.D. Truong, D. Kadmon, B.K. McCune, K.C. Flanders, P.T. Scardino, T.C. Thompson, Association of transforming growth factor-beta 1 with prostate cancer: an immunohistochemical study, Hum. Pathol. 24 (1993) 4–9.