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Research paper

Culture supernatants of cervical cancer cells induce an M2 phenotypic profile in THP-1 macrophages

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ABSTRACT

Patients with cervical cancer (CxCa) typically present an infiltrate of tumor-associated macrophages, which is associated with a poor prognosis. We found that CxCa cell lines (HeLa, SiHa, and C-33A) secreted factors involved in regulating tumor growth including IL-6, IL-4, $PDGF_{AA}$, HGF, VEGF, ANG-2, and TGF-β3. We assessed the effects of culture supernatants from these cell lines on macrophages derived from the THP-1 cell line. Macrophages treated with culture supernatants from CxCa cells developed an M2-like phenotype with expression of CD163, low nitric oxide release, and high secretion of IL-6, $PDGF_{AA}$, HGF, ANG-2, and VEGF. The macrophages continued to produce $PDGF_{AA}$, $PDGF_{BB}$, and VEGF 48 h after the CxCa cell culture supernatants were removed. The induction of M2 macrophages *in vivo* favors tumor growth, angiogenesis, tissue remodeling, and metastasis. These results demonstrated that factors secreted by CxCa cells induced a stable M2 phenotype in THP-1 macrophages.

1. Introduction

Cervical cancer (CxCa) is the second most commonly diagnosed and third leading cause of cancer death among females in developing countries [1]. The main etiological factor for the development of CxCa is persistent infection with high-risk human papillomavirus (HPV) genotypes such as HPV-16 and HPV-18 [2–4]. However, other associated co-factors including multiparity, smoking, long-term consumption of oral contraceptive pills, and immunosuppression have also been implicated in the development of CxCa [5,6].

An immunosuppressive microenvironment is produced in diverse cancers through low immunogenicity and the production of cytokines such as transforming growth factor (TGF)-β, interleukin (IL)-10, and indoleamine 2,3-dioxygenase that suppress pro-inflammatory Th1 and cytotoxic lymphocyte responses. However, in approximately 50% of patients with CxCa, a weak proliferative response of T-cells has been observed. This is associated with a phenotypic switch of tumor-infiltrated cells such as T cells, neutrophils, dendritic cells, and macrophages from tumor-suppressing to tumor-promoting behaviors in response to the tumor microenvironment [7–9]. In addition, diverse reports have described the overexpression of chemokines such as monocyte chemoattractant protein-1 in CxCa [10–12] and a high level of infiltration of macrophages into the tumor tissue [7,13].

Abbreviations: ANG, angiopoietin; CMS, culture medium supplement; CxCa, cervical cancer; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; GM-CSF, granulocyte-monocyte colony-stimulating factor; HGF, hepatocyte growth factor; HLA-DR, human leukocyte antigen-antigen D related; HPV, human papillomavirus; IFN, interferon; IL, interleukin; LPS, lipopolysaccharides; M-CSF, macrophage-colony stimulating factor; MFI, mean fluorescence intensity; NO, nitric oxide; PBS, phosphate-buffered saline; PDGF, platelet-derived growth factor; PMA, phorbol 12-myristate 13-acetate; RPMI, Roswell Park Memorial Institute medium; TGF, transforming growth factor; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor.

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Macrophages that infiltrate into the tumor are generally called tumor-associated macrophages and mainly exhibit two contrasting phenotypes. First, classically activated or M1 macrophages that are activated by interferon (IFN)-γ, lipopolysaccharides (LPS) through Toll-like receptors, and granulocyte-monocyte colony-stimulating factor (GM-CSF). Macrophages of the M1 phenotype are characterized by a high expression of major histocompatibility complex class II and co-stimulatory molecules such as CD86/CD80, as well as a high production of IL-12, IL-23, tumor necrosis factor (TNF)-α, reactive oxygen species, and reactive nitrogen species such as nitric oxide (NO). The main functions of M1 macrophages are to kill intracellular pathogens, destroy tumors, and promote the Th1 immune response. Second, alternatively activated or M2 macrophages that are activated by IL-4, IL-13, IL-10, IL-33, and IL-21 and are characterized by the expression of CD163 and CD206. Macrophages of the M2 phenotype participate in parasite clearance, tissue remodeling, wound healing, immunoregulation, promoting the Th2 immune response, and tumor promotion through the expression of vascular endothelial growth factor (VEGF), transforming growth factor (TGF)-β, indoleamine 2,3-dioxygenase, and programmed death ligand 1 expression. Tumor-associated macrophages have a high plasticity and frequently share features of both the M1 and M2 phenotypes. In diverse tumors, the presence of M2 macrophages is associated with a poor prognosis [14–16].

Several reports have described the secretion of various factors including IL-6, IL-13, TGF-β, VEGF, and prostaglandin E_2 by CxCa cells [11,17–19]. However, it has not yet been clearly established whether factors secreted by CxCa cells perform an important role in the induction or maintenance of the M2 macrophage phenotype.

Therefore, in this work, we first characterized the profile of cytokines, growth factors, and NO present in the CxCa cell culture supernatants. Then, we investigated whether those supernatants could induce the M2 phenotype in THP-1 macrophages. Finally, we evaluated whether the M2 phenotype was maintained after removing the CxCa cell culture supernatants.

2. Methods and materials

2.1. Reagents and antibodies

Dulbecco's modified Eagle's medium (DMEM), Roswell Park Memorial Institute-1640 medium (RPMI-1640), heat-inactivated fetal bovine serum (FBS), L-glutamine, penicillin/streptomycin antibiotics solution, and phosphate-buffered saline (PBS) were purchased from Life Technologies (Carlsbad, CA, USA). Phorbol 12-myristate 13-acetate (PMA) and LPS were purchased from Sigma-Aldrich (St. Louis, MO, USA). IL-4, IL-10, and IFN-γ were purchased from BioLegend (San Diego, CA, USA). For flow cytometry assays, the following anti-human monoclonal antibodies were employed: CD14-PerCP, CD80-FITC, CD86-PE, CD163-FITC, and CD206-PECy7 (BioLegend), and human leukocyte antigen-antigen D related (HLA-DR)-APC, STAT1-PE, pSTAT1-PE, STAT6-PE, pSTAT6-PE, and NF-κB p65-PE (BD Biosciences, San Jose, CA, USA). For the determination of cytokines and growth factors in the cell culture supernatants, the Human Th1/Th2/Th17 CBA kit (BD Biosciences) and Human Growth Factor Panel Multi-analyte Flow Assay kit (BioLegend) were utilized. For the determination of TGF-β, the Bio-Plex® Pro TGF-β-3-plex Assay (Bio-Rad Laboratories, Hercules, CA, USA) was employed. NO production was measured using the Total Nitric Oxide Nitrate/Nitrite Colorimetric Assay (R&D Systems, Minneapolis, MN, USA).

2.2. Cell culture

HeLa (HPV-18$^+$), SiHa (HPV-16$^+$), and C-33A (HPV$^-$) CxCa cell lines were kindly provided by Dr. Boukamp (DKFZ, Heidelberg, Germany) and were propagated *in vitro* by culture in DMEM containing culture medium supplement (CMS; 10% [v/v] heat-inactivated FBS, 2 mM L-glutamine, and 1 × streptomycin/penicillin solution). THP-1 monocytes (cat. no. TIB-202) were purchased from American Type Culture Collection (Manassas, VA, USA) and were propagated *in vitro* by culture in RPMI-1640 containing CMS. All cell lines were incubated at 37 °C in a humidified atmosphere containing 5% CO_2.

2.3. Collection of culture medium supernatants from CxCa cell lines

CxCa cell lines were independently seeded in 6-well culture plates at 1×10^5 cells per 3 mL of DMEM containing CMS per well. At day 5 of cell culture, the culture medium supernatants were collected [17] and stored at −80 °C until they were employed for the assessment of M2 macrophage induction and the release of cytokines, growth factors, and NO.

2.4. THP-1 cell line differentiation and treatment with CxCa cell culture supernatants

To differentiate the THP-1 cell line into macrophages, the undifferentiated THP-1 cells were seeded onto 12-well culture plates at 1×10^6 cells per 2 mL of culture medium (RPMI containing CMS and 200 nM PMA) and cultured for 3 d. Subsequently, the cells were washed 3 times with PBS and treated with CxCa cell culture supernatants (50% of the total cell culture volume) for either 1 h or 3 d. In the THP-1 macrophages treated for 1 h with CxCa cell culture supernatants, we assessed the phosphorylation of STAT1, STAT6, and NF-κB (subunit p65) transcription factors. In the THP-1 macrophages treated for 3 d with CxCa cell culture supernatants, we evaluated the expression of membrane receptors (CD14, CD80, CD86, HLA-DR, CD163, and CD206) and the concentrations of cytokines, NO, TGF-β family proteins, and growth factors in the macrophage culture medium. In another group of THP-1 macrophages, after treatment with CxCa cell culture supernatants for 3 d, we replaced the culture medium with fresh medium, cultivated the cells for an additional 48 h, and then evaluated the concentrations of cytokines, NO, TGF-β family proteins, and growth factors in the culture medium.

As an experimental control, we activated THP-1 macrophages with 20 ng/mL of IFN-γ plus 100 ng/mL of LPS or 20 ng/mL of IL-4 plus 20 ng/mL of IL-10 to induce the M1 and M2 phenotypes, respectively. The cells were incubated with these cytokines during the final 18 h of treatment with 200 nM PMA. Untreated Mθ macrophages (baseline condition without phenotypic induction) were maintained in RPMI containing CMS during the assays, and M1 and M2 macrophages were also maintained in RPMI containing CMS after activation.

2.5. Assessment of CD14, CD80, CD86, CD163, CD206, and HLA-DR expression, and STAT1, p65, and STAT6 phosphorylation in THP-1 macrophages by flow cytometry

To evaluate the expression of CD14, CD80, CD86, CD163, CD206, and HLA-DR in THP-1 macrophages treated for 3 d with the CxCa cell culture supernatants, we harvested the cells in all experimental groups, washed them twice with PBS, and stained them to discriminate live and dead cells with the LIVE/DEAD® Fixable Near-IR Dead Cell Stain kit (Life Technologies) according to the manufacturer's protocol. The harvested cells were then washed twice with PBS, and human Fc receptors were blocked using Fc Receptor Blocking Solution (BioLegend). We then incubated the cells with antibodies for human CD163-FITC, CD80-FITC, CD86-PE, CD14-PerCP, CD206-PECy7, or HLA-DR-APC for 30 min at room temperature. Subsequently, we washed the cells twice with PBS,

resuspended them in PBS, and then analyzed them by flow cytometry. Labeling of STAT1, NF-κB p65, and STAT6 phosphorylation was performed as follows: in brief, the cells were fixed using Fixation Buffer (BD Biosciences) for 15 min at 4 °C. Then, they were washed twice with staining buffer (PBS, 0.09% [w/v] sodium azide, and 0.2% [v/v] FBS), and then permeabilized with permeabilization buffer (90% [v/v] methanol and 10% [v/v] PBS) for 30 min on ice. The cells were then washed 3 times with staining buffer, incubated with antibodies against STAT1-PE, pSTAT1-PE, NF-κB p65-PE, STAT6-PE, or pSTAT6-PE for 30 min at 4 °C in the dark, and washed once more with staining buffer. Finally, the cells were resuspended in staining buffer and analyzed by flow cytometry. We acquired 10,000 events corresponding to live cells per experimental group as assessed using the LIVE/DEAD® assay and an Attune™ flow cytometer (Life Technologies). An appropriate isotype and fluorescence minus one (FMO) controls were utilized to adjust for background fluorescence. Results were analyzed using FlowJo V10-1r5 (Tree Star, Ashland, OR, USA) and reported as geometric mean fluorescence intensity (MFI).

2.6. Quantification of cytokines and growth factors in cell culture supernatants by flow cytometry

After 3 d of treatment of THP-1 macrophages with CxCa cell culture supernatants with or without an additional 48 h of culture in fresh medium, we collected the culture media from all experimental groups. The concentrations of IL-17, IFN-γ, TNF-α, IL-10, IL-6, IL-4, and IL-2 were evaluated using the Human Th1/Th2/Th17 Cytometric Bead Array kit (BD Biosciences) according the manufacturer's protocol. For the assessment of growth factors in the cell culture supernatants, we utilized the Human Growth Factor Panel Multi-analyte Flow Assay kit (BioLegend) according to the procedure recommend by the manufacturer. The growth factors evaluated were angiopoietin-2 (ANG-2), epidermal growth factor (EGF), erythropoietin (EPO), fibroblast growth factor (FGF)-basic, granulocyte-colony stimulating factor (G-CSF); GM-CSF; hepatocyte growth factor (HGF); macrophage-colony stimulating factor (M-CSF); platelet-derived growth factor $(PDGF)_{AA}$; $PDGF_{BB}$; stem cell factor (SCF); TGF-α, and VEGF. Cytokine and growth factor concentrations were expressed in pg/mL.

2.7. Determination of TGF-β in cell culture supernatants

After 3 d of treatment of THP-1 macrophages with CxCa cell culture supernatants with or without an additional 48 h of culture in fresh medium, we collected the culture media from all experimental groups. The production of the three isoforms of the TGF-β family (TGF-β1, TGF-β2, and TGF-β3) was evaluated utilizing the Bio-Plex Pro™ TGF-β-3-Plex Assay (Bio-Rad Laboratories) according to the manufacturer's protocol. Data were acquired employing a Bio-Plex® 200 System (Bio-Rad Laboratories) and analyzed with Bio-Plex® Manager ver. 6.0 statistical software, and TGF-β levels were expressed in pg/mL. To normalize the concentration of TGF-β in all groups, we evaluated the concentration of TGF-β in each CxCa cell culture supernatant, and the value obtained for the corresponding supernatant was subtracted from that measured in each macrophage culture medium sample.

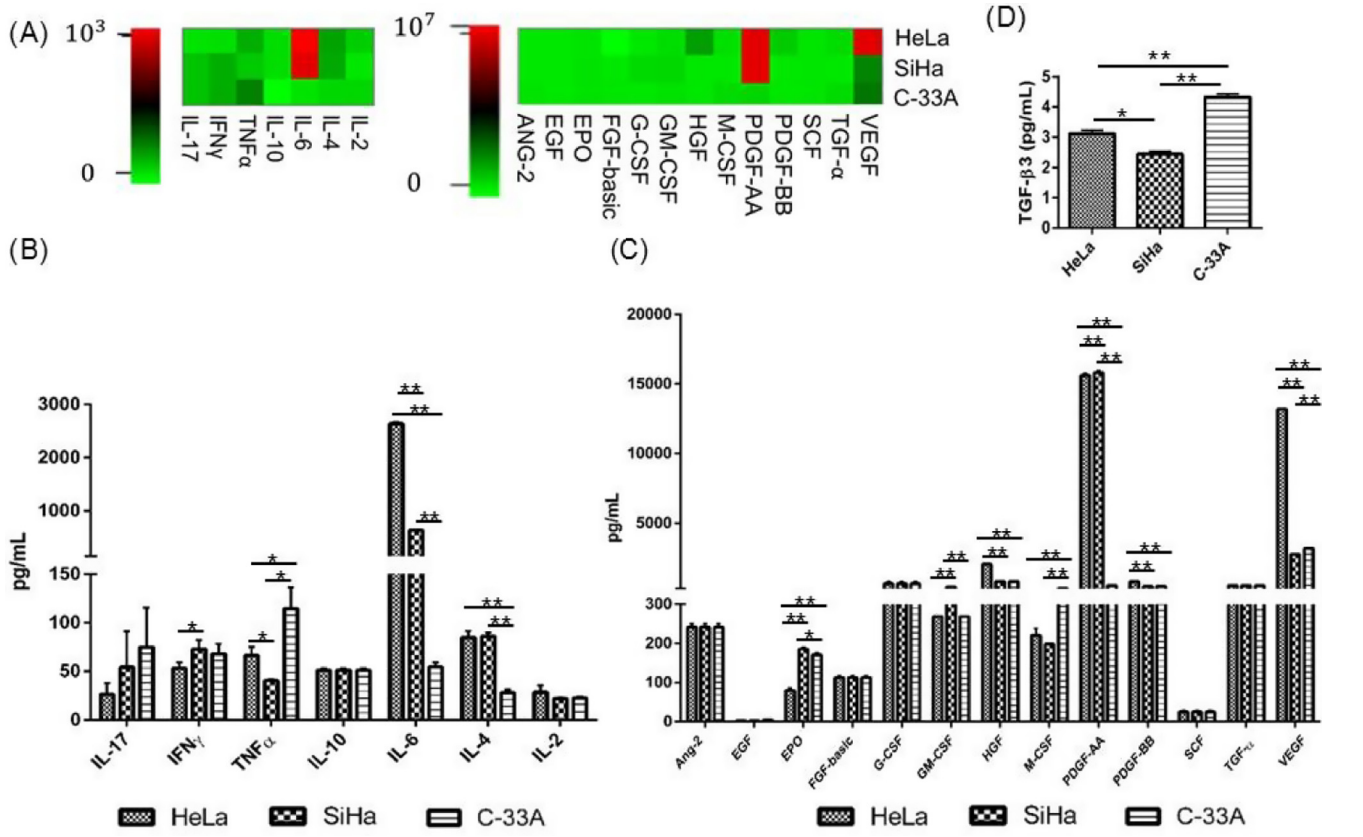

Fig. 1. Cytokines, growth factors, and TGF-β proteins in the supernatants of HeLa, SiHa, and C-33A cell cultures. (A) Heatmaps showing the relative concentrations of Th1/Th2/Th17 cytokines and growth factors secreted into the supernatants of HeLa, SiHa, and C-33A cell cultures at day 5 of cell culture. (B) Graph showing the profiles of cytokines secreted by HeLa, SiHa, and C-33A cells. (C) Graph showing the profiles of growth factors secreted by HeLa, SiHa, and C-33A cells. (D) TGF-β3 secreted into the culture supernatants of HeLa, SiHa, and C-33A cells. Unpaired Student's *t* tests were performed to compare group means. $^{*}p < 0.05$ and $^{**}p < 0.01$. Data are shown as the mean ± standard deviation (SD) of three independent experiments carried out in triplicate.

2.8. Quantification of NO in cell culture supernatants by colorimetric assay

In the culture media of all experimental groups, we assessed NO production using the Total Nitric Oxide Nitrate/Nitrite Colorimetric Assay (R&D Systems) according to the procedure recommended by the supplier. Finally, optical densities were read at 540 nm with wavelength correction at 690 nm utilizing a Synergy™ HT Multi-mode Microplate Reader (BioTek Instruments, Winooski, VT, USA) and the results were expressed in μmol/L. To normalize the results for NO production in all groups, we evaluated NO production in each CxCa cell culture supernatant, and the value obtained for the corresponding supernatant was subtracted from that measured in each macrophage culture medium sample.

2.9. Statistical analysis

Statistical analysis was performed using GraphPad Prismy® ver. 6 software (GraphPad, La Jolla, CA, USA). Data are presented as the mean ± standard deviation of at least three independent experiments carried out in triplicate. Student's *t* test was utilized for comparisons between groups. Values of $p < 0.05$ were considered significant.

3. Results

3.1. Profile of soluble factors secreted by CxCa cells

Cancer cells release several factors that contribute to promoting cellular proliferation and suppressing the immune response. For this reason, we evaluated the profile of soluble factors present in the culture supernatants of HeLa, SiHa, and C-33A cells. Fig. 1A shows heatmaps that represent the relative concentrations of cytokines and growth factors detected in the CxCa cell culture supernatants. The three CxCa exhibited distinct profiles of secreted factors. IL-6 was secreted in greater quantities by HeLa and SiHa cells ($p < 0.01$) in comparison to C-33A cells. The same tendency was observed for IL-4 production. However, C-33A cells secreted more TNF-α than HeLa and SiHa cells did ($p < 0.05$, Fig. 1B). Likewise, we found that HeLa cells secreted higher amounts of HGF and VEGF than SiHa and C-33A cells did ($p < 0.01$). PDGF-$_{AA}$ was secreted at higher concentrations by HeLa and SiHa cells ($p < 0.01$) in comparison to C-33A cells. SiHa cells showed a higher secretion of GM-CSF than HeLa and C-33A cells did ($p < 0.01$), while M-CSF was produced in greater quantities by C-33A cells ($p < 0.01$, Fig. 1C). Additionally, TGF-β3 was secreted at a low concentration by CxCa cell lines, and we observed that C-33A cells released the

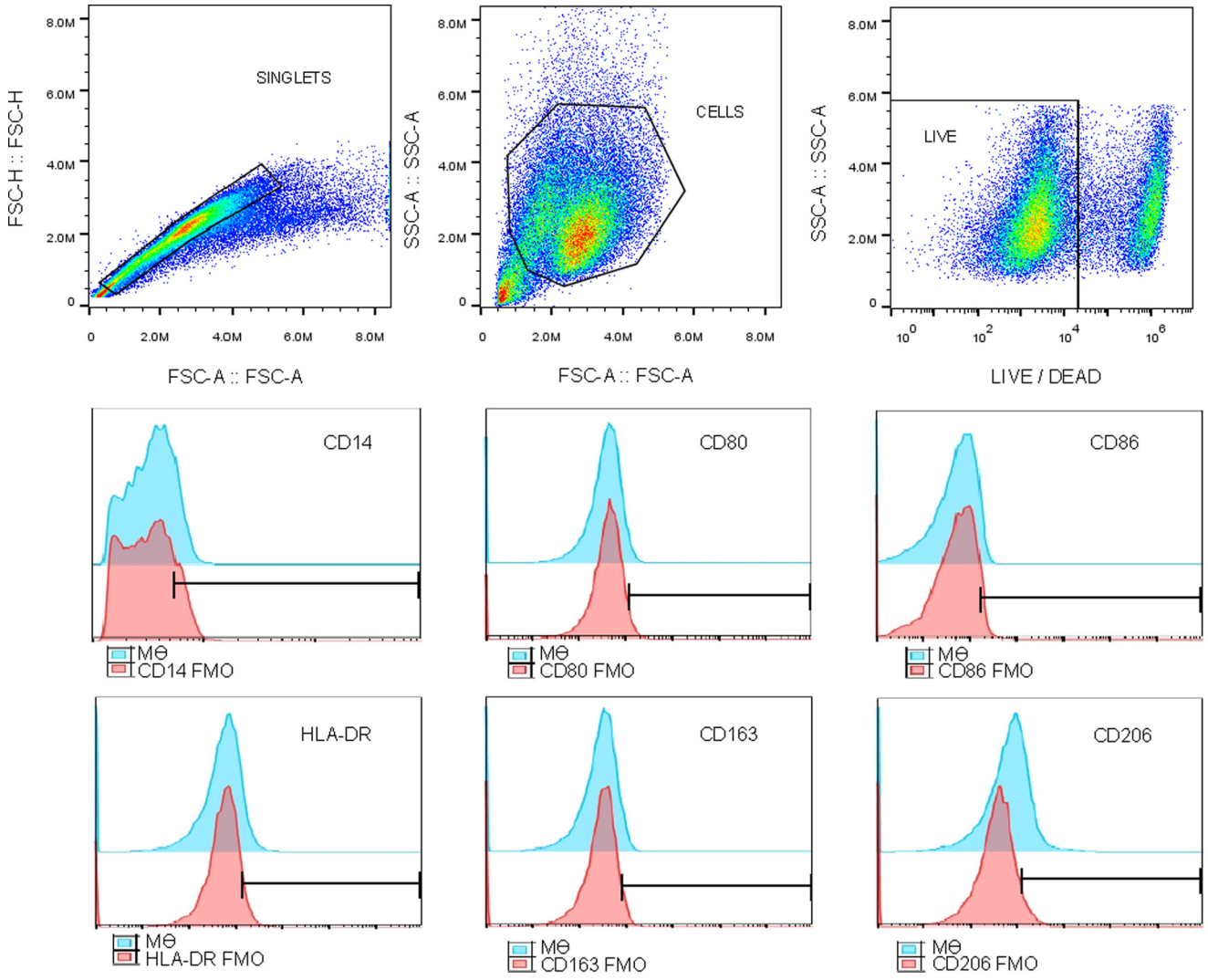

Fig. 2. Analysis scheme for the determination of membrane receptors in THP-1 macrophages stimulated with cervical cancer (CxCa) cell culture supernatants. Representative strategy for CD14, CD80, CD86, HLA-DR, CD163, and CD206 determination in THP-1 macrophages treated with CxCa cell culture supernatants. The control groups were MΘ macrophages (baseline phenotype) and fluorescence minus one (FMO) negative controls.

highest concentration of TGF-β3 among the three cell lines evaluated ($p < 0.01$, Fig. 1D). We did not observe TGF-β1 or TGF-β2 in CxCa cell culture supernatants. Based on these findings, we can conclude that CxCa cell lines secrete several factors that have been implicated in proliferation and angiogenesis.

3.2. Effects of CxCa cell culture supernatants on the expression of surface receptors and transcription factors by THP-1 macrophages

To evaluate the effects of CxCa cell culture supernatants on the polarization of THP-1 macrophages, we assessed the expression of receptors that are differentially expressed between the M1 and M2 phenotypes such as CD14, CD80, CD86, CD163, CD206, and HLA-DR in macrophages treated for 3 d with CxCa cell culture supernatants. In Fig. 2, we show our strategy for determining the expression of membrane receptors in macrophages. THP-1 macrophages treated with HeLa, SiHa, and C-33A cell culture supernatants did not exhibit changes in CD86 expression in comparison to that of the Mθ control group (Table 1). These results are similar to those that we obtained by quantitative reverse transcriptase-polymerase chain reaction (Fig. S1). However, the expression of receptors related to M1 macrophages, such as CD80 and HLA-DR, was increased only in the macrophages treated with C-33A cell culture supernatants ($p < 0.05$) and was higher than that observed in the M1 control group (Table 1).

On the other hand, the CD163 and CD206 receptors are typically expressed by M2 macrophages. In our experiments, we observed increased CD163 expression in all THP-1 macrophages treated with CxCa cell culture supernatants to a higher level than that in the Mθ, M1, and M2 macrophage control groups ($p < 0.01$). THP-1 macrophages treated with SiHa and C-33A cell culture supernatants exhibited an increased MFI for CD206 expression in comparison with that of Mθ macrophages ($p < 0.01$). CD14 expression in THP-1 macrophages treated with CxCa cell culture supernatants increased in comparison with that in Mθ macrophages (Table 1).

Next, we assessed the expression of transcription factors involved in the induction of the M1 phenotype, such as STAT1 and p65, or the M2 phenotype, such as STAT6, in THP-1 macrophages. Our findings showed that the expression of the active form of STAT1 (phosphorylated STAT1 [pSTAT1]) was similar between THP-1 macrophages treated with CxCa cell culture supernatants and the M2 control group, and was less than that observed in the M1 control group (Table 1 and Fig. 3). However, we did not observe differences in pSTAT6 expression between macrophages treated with CxCa cell culture supernatants and the control groups (Table 1). Based on these results, we concluded that all CxCa cell culture supernatants induced CD163 expression in the THP-1 macrophages, and C-33A cell culture supernatants specifically induced the expression of M1 receptors such as CD80 and HLA-DR, although the induction of this M2-like phenotype did not appear to be mediated through the activation of STAT6.

3.3. Cytokines, growth factors, and NO production by THP-1 macrophages after treatment with CxCa cell culture supernatants

To determine the effects of CxCa cell culture supernatants on the release of cytokines, growth factors, and NO by THP-1 macrophages, we assessed the secretion of these molecules by THP-1 macrophages treated for 3 d with HeLa, SiHa, or C-33A cell culture supernatants. The heatmaps presented in Fig. 4A show the relative concentrations of cytokines and growth factors that were released by all experimental groups. We observed that M1 macrophages mainly secreted IFN-γ and IL-2, while M2 macrophages produced IL-10 and IL-4 ($p < 0.05$, Fig. 4B). Interestingly, THP-1

Table 1
Expression of membrane receptors and transcription factors related with M1 and M2 phenotype of macrophages. Membrane receptors and transcription factors are presented in units of MFI. The control groups were Mθ macrophages (baseline phenotype), M1 macrophages (treated with IFN-γ + LPS), and M2 macrophages (treated with IL-4 + IL-10). sHeLa, sSiHa, and sC-33A represent the supernatants of three different CxCa cell lines. Unpaired Student's *t* tests were performed to compare group means. $^{*}p < 0.05$ and $^{**}p < 0.01$. Data are shown as the mean ± standard deviation of three independent experiments carried out in triplicate.

	CD14 (MFI)	CD80 (MFI)	CD86 (MFI)	HLA-DR (MFI)	CD163 (MFI)	CD206 (MFI)	p65 (MFI)	STAT1 (MFI)	pSTAT1 (MFI)	STAT6 (MFI)	pSTAT6 (MFI)
Mθ	68,820 ± 578.60	1603.67 ± 46.11	177.67 ± 0.58	1807.33 ± 8.96	1035 ± 3.46	1768 ± 7.00	468.67 ± 33.86	497.33 ± 15.14	500 ± 26.85	651.67 ± 9.50	431.33 ± 40.45
M1	73730.33 ± 273.12	1693.67 ± 18.90	175 ± 2.65	2047.33 ± 3.79	1072.67 ± 5.13	1836 ± 17.35	481 ± 36.51	505.33 ± 14.36	**867 ± 5.57****	652 ± 23.07	431 ± 34.83
M2	72875.67 ± 109.87	1654.33 ± 20.53	175 ± 1.00	1920.67 ± 16.86	1091.67 ± 4.04	1837.33 ± 34.67	465 ± 35.16	481 ±15.72	485.67 ± 24.54	548.67 ± 14.22	436.33 ± 34.96
Mθ + sHeLa	**72149.33 ± 393.16****	1695.67 ± 30.86	176.33 ± 1.15	**1745 ± 3.46****	**1100 ± 13.08****	1782.67 ± 33.08	467.67 ± 33.08	491.67 ± 15.50	485.33 ± 24.00	518.13 ± 38.73	433.67 ± 37.63
Mθ + sSiHa	**70494.33 ± 341.63***	1675.33 ± 18.88	176.33 ± 0.58	1826.33 ± 11.37	**1093.33 ± 5.13****	**1835.33 ± 14.84****	469.33 ± 33.38	495 ± 14.00	485 ± 26.89	520.67 ± 24.50	436.33 ± 43.32
Mθ + sC-33A	69,546 ± 159.91	**1757 ± 28.16***	176.67 ± 1.53	**2051 ± 7.81****	**1133.67 ± 8.33****	**1856.33 ± 19.35****	474.67 ± 34.96	487.33 ± 14.36	476.33 ± 23.97	511.67 ± 23.00	447.67 ± 43.15

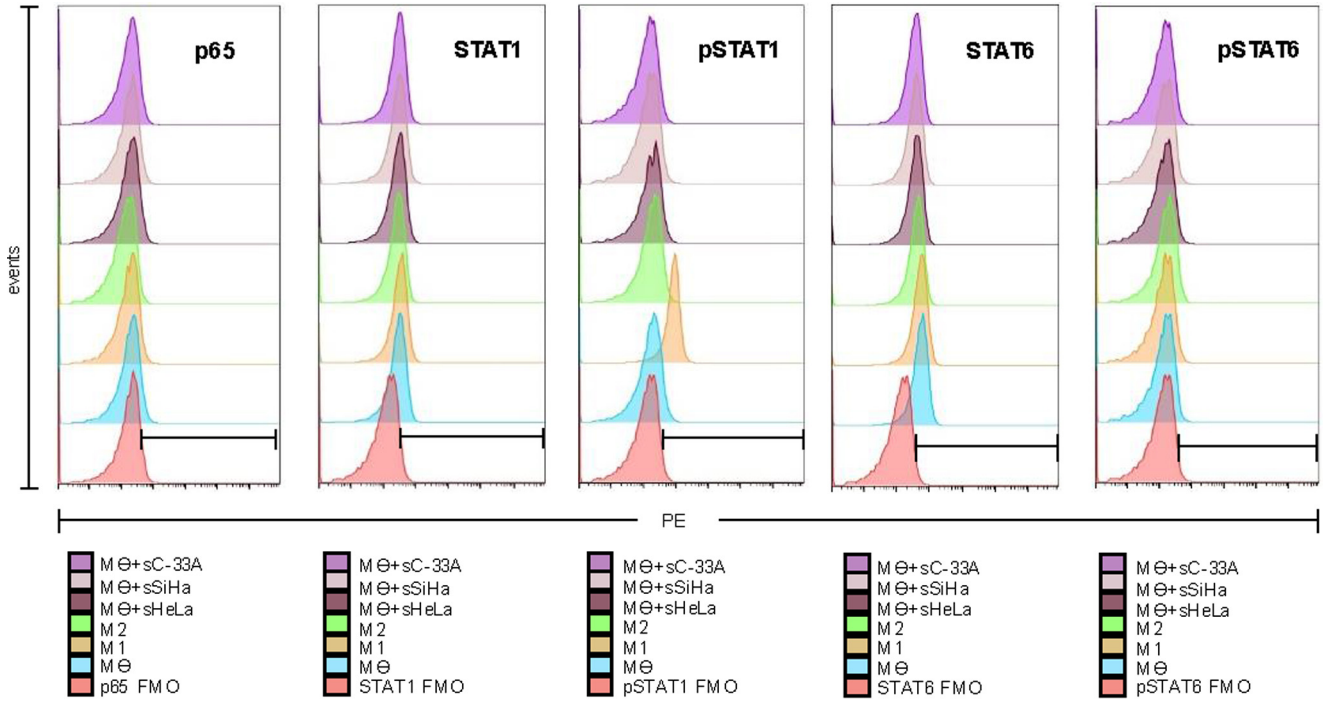

Fig. 3. STAT1, pSTAT1, NF-κB p65, STAT6, and pSTAT6 expression in THP-1 macrophages treated for 1 h with HeLa, SiHa, and C-33A cell culture supernatants. p65, total STAT1, pSTAT1, total STAT6, and pSTAT6 expression in THP-1 macrophages treated for 1 h with HeLa, SiHa, and C-33A cell culture supernatants under baseline conditions (Mθ) or with M1 phenotype induction (treated with IFN-γ + LPS) or M2 phenotype induction (treated with IL-4 + IL-10). Representative images are shown of three independent experiments carried out in triplicate. "p": phosphorylated state.

macrophages treated with HeLa and SiHa cell culture supernatants released high levels of IL-6 in comparison with that released by Mθ, M1, and M2 macrophages ($p < 0.05$, Fig. 4B).

THP-1 macrophages treated with HeLa and SiHa cell culture supernatants secreted larger amounts of HGF, $PDGF_{AA}$ ($p < 0.01$), and ANG-2 ($p < 0.05$) than Mθ and M1 macrophages did. Furthermore, HeLa cell culture supernatants induced an increase in VEGF production by THP-1 macrophages ($p < 0.01$). Finally, THP-1 macrophages treated with C-33A cell culture supernatants released increased amounts of ANG-2, HGF, and SCF ($p < 0.01$) in comparison with the release of these factors by Mθ and M1 macrophages (Fig. 4C).

We evaluated the concentrations of members of the TGF-β protein family in the culture media of THP-1 macrophages treated with CxCa cell culture supernatants. HeLa, SiHa, and C-33A cell culture supernatants induced an increment of TGF-β3 secretion in comparison with that of Mθ and M1 macrophages ($p < 0.05$, Fig. 4D). Moreover, NO production was suppressed in all groups treated with HeLa, SiHa, and C-33A cell culture supernatants ($p < 0.01$, Fig. 4E). These results suggest that exposure to the CxCa cell culture supernatants led to the THP-1 macrophages undergoing polarization towards a phenotype that would be expected to promote tumor growth and angiogenesis, and also impair the anti-tumor response of the macrophages.

3.4. Sustained production of growth factors by THP-1 macrophages 48 h after the removal of CxCa cell culture supernatants

We aimed to evaluate whether THP-1 macrophages treated with CxCa cell culture supernatants for 3 d maintained their increased production of IL-6, $PDGF\text{-}_{AA}$, HGF, VEGF, ANG-2, and TGF-β3 and reduced release of NO after removing the CxCa cell supernatants. Therefore, we treated THP-1 macrophages with HeLa, SiHa, or C-33A cell culture supernatants for 3 d and then maintained the cells for an additional 48 h in fresh medium without the CxCa cell culture supernatants. Then, the concentrations of cytokines, growth factors, TGF-β, and NO in the macrophage cell culture medium were evaluated. The heatmaps presented in Fig. 5A depict the relative concentrations of cytokines and growth factors in the culture media of all experimental groups. The data presented in Fig. 5B show an increased release of IFN-γ and IL-2 by THP-1 macrophages previously treated with SiHa and C-33A cell culture supernatants in comparison with that of Mθ and M1 macrophages ($p < 0.05$). The release of IFN-γ by those two groups of macrophages was similar to that observed in the M2 control group. IL-17 was principally secreted by THP-1 macrophages previously treated with the SiHa cell culture supernatant ($p < 0.05$). The most abundant growth factor detected in the THP-1 macrophage cell culture media was HGF ($p < 0.05$) (Fig. 5C). The concentration of GM-CSF was also diminished in all THP-1 macrophages previously treated with HeLa, SiHa, and C-33A cell culture supernatants compared with that in Mθ and M1 macrophages ($p < 0.05$). M-CSF was increased in the groups prestimulated with HeLa and SiHa cell culture supernatants ($p < 0.01$). Interestingly, $PDGF_{AA}$ was elevated only in the groups previously treated with HeLa and SiHa cell culture supernatants ($p < 0.01$), while in the group previously treated with the C-33A cell culture supernatant, the concentration of $PDGF_{BB}$ was increased ($p < 0.01$), in comparison with all other groups. VEGF production was augmented exclusively in the group pretreated with the HeLa cell culture supernatant ($p < 0.01$) (Fig. 5C).

When we assessed the presence of TGF-β and NO in THP-1 macrophage cell culture supernatants, we observed that TGF-β3 was increased in all of the groups previously treated with the cell culture supernatants of CxCa cancer cells in comparison with that of Mθ and M1 macrophages (Fig. 5D). NO production only remained significantly suppressed in the group previously treated with the HeLa cell culture supernatant and in M2 macrophages ($p < 0.05$) (Fig. 5E).

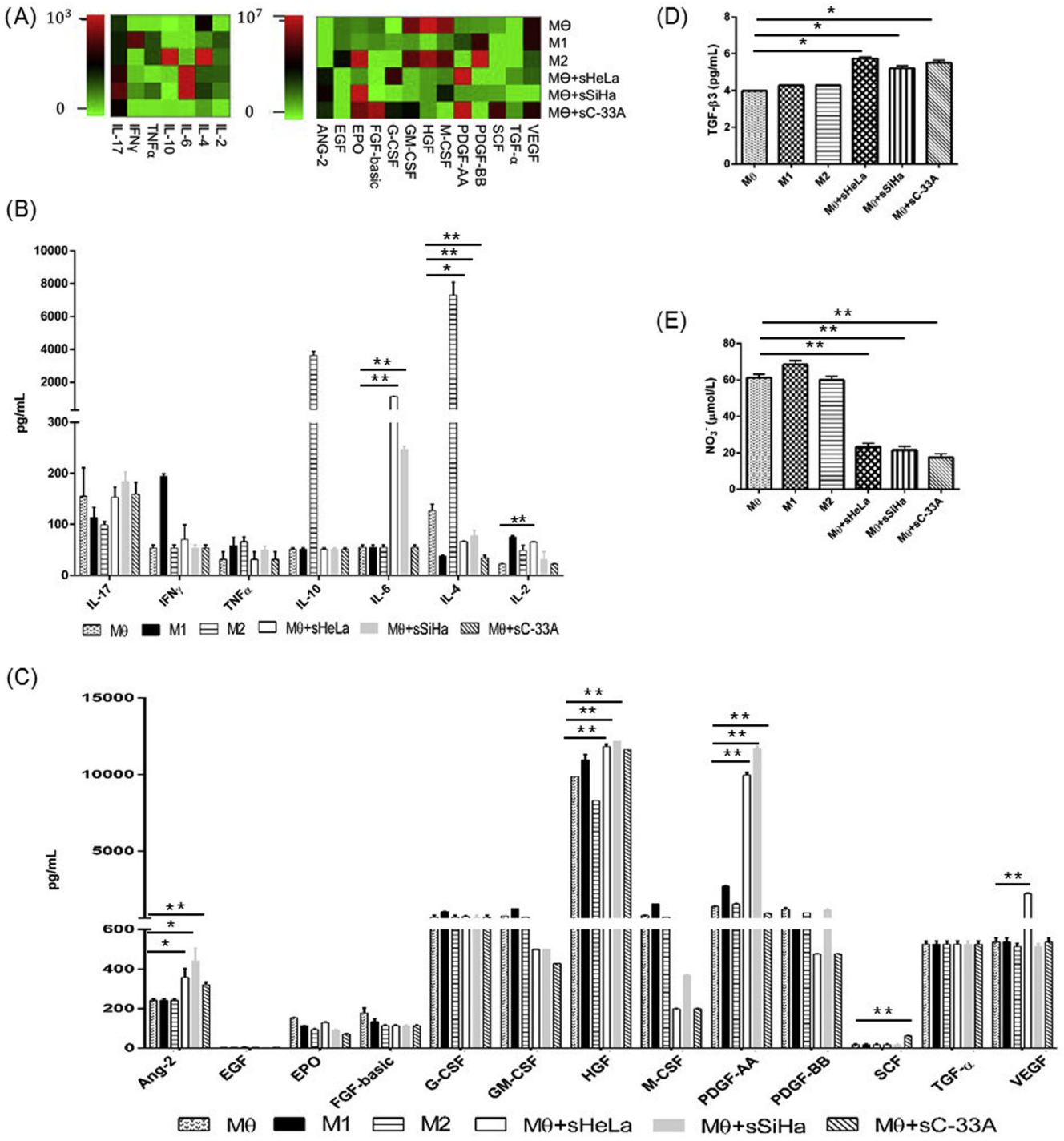

Fig. 4. Cytokine profiles, growth factors, TGF-β proteins, and nitric oxide (NO) in the culture media of THP-1 macrophages treated for 3 d with HeLa, SiHa, and C-33A cell culture supernatants. (A) Heatmaps of the cytokines and human growth factor profiles in the cell culture supernatants of THP-1 macrophages treated with HeLa, SiHa, and C-33A cell culture supernatants for 3 d. (B) Graph showing the profiles of cytokines secreted by THP-1 macrophages after treatment with HeLa, SiHa, and C-33A cell culture supernatants. (C) Graph showing the profiles of human growth factors secreted by THP-1 macrophages after treatment with HeLa, SiHa, and C-33A cell culture supernatants. (D) TGF-β3 secreted into the culture medium of THP-1 macrophages treated with HeLa, SiHa, and C-33A cell culture supernatants. (E) NO production by THP-1 macrophages treated for 3 d with HeLa, SiHa, and C-33A cell culture supernatants. The control groups were Mθ macrophages (baseline phenotype), M1 macrophages (treated with IFN-γ + LPS), and M2 macrophages (treated with IL-4 + IL-10). sHeLa, sSiHa, and sC-33A represent the culture supernatants of three different CxCa cell lines. Unpaired Student's *t* tests were performed to compare group means. $^{*}p < 0.05$ and $^{**}p < 0.01$. Data are shown as the mean ± SD of three independent experiments carried out in triplicate.

4. Discussion

To elucidate the different strategies that tumors employ to induce an immunosuppressive microenvironment that promotes tumor growth and metastasis, it is necessary to understand how cancer cells influence immune cells. Previously, our work group described the presence of IL-6 and IL-13 in CxCa cell culture supernatants and a high expression of CD163 in U937 macrophages after they were treated with CxCa cell culture supernatants [18]. In the present work, we assessed the diverse secreted factors in cell

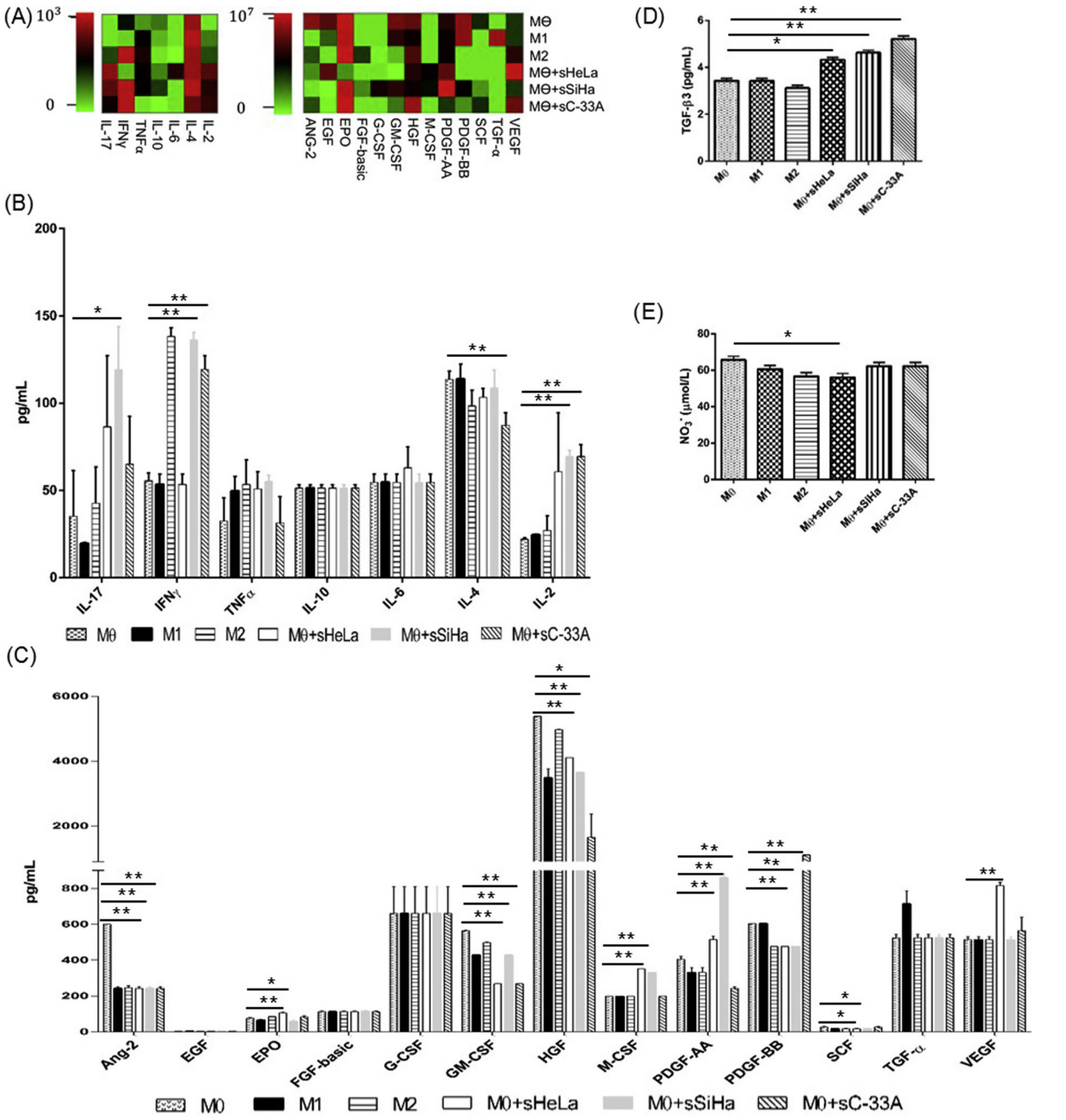

Fig. 5. Cytokines, growth factors, TGF-β proteins, and NO in the culture media of THP-1 macrophages maintained for an additional 48 h in fresh medium after treatment for 3 d with HeLa, SiHa, and C-33A cell culture supernatants. (A) Heatmaps showing the profiles of cytokines and human growth factors in the culture media of THP-1 macrophages. (B) Graph showing the profiles of cytokines secreted by THP-1 macrophages. (C) Graph showing the profiles of growth factors secreted by THP-1 macrophages. (D) TGF-β3 in the culture media of THP-1 macrophages. (E) NO production by THP-1 macrophages. The control groups were MΘ macrophages (baseline phenotype), M1 macrophages (treated with IFN-γ + LPS), and M2 macrophages (treated with IL-4 + IL-10). sHeLa, sSiHa, and sC-33A represent 3-day pretreatments with the cell culture supernatants of three different CxCa cell lines. Unpaired Student's *t* tests were performed to compare group means. $^{*}p < 0.05$ and $^{**}p < 0.01$. Data are shown as the mean ± SD of three independent experiments carried out in triplicate.

culture supernatants from the HeLa (HPV-18^{+}), SiHa (HPV-16^{+}), and C-33A (HPV^{-}) CxCa cell lines, and their effects on the induction of the M2 phenotype in THP-1 macrophages. We detected high concentrations of IL-6 and IL-4 in HeLa and SiHa cell culture supernatants, which is consistent with previously reported results on CxCa cells [11,17]. In the C-33A cell culture supernatants, we observed a predominant secretion of TNF-α and IL-17. These differences between the three CxCa cell lines could be explained by their different mechanisms of transformation (i.e., HPV^{+} vs. HPV^{-}). C-33A cells do not possess the HPV E6 and E7 oncoproteins that inhibit TNF responses [20]; therefore, C-33A might produce a high level of IL-17 as an immunoevasion mechanism. It has been

described that a high production of IL-17 induces cyclooxygenase-2 expression through the ERK1/2 and NF-κB pathways, which promotes prostaglandin E_2 synthesis and secretion by the tumor cell [21]. Our results suggested that the induction of THP-1 macrophage polarization towards the M2-like phenotype by the CxCa cell culture supernatants could perhaps be due to the combined action of multiple soluble factors present in these supernatants.

In HeLa and SiHa cell culture supernatants, we observed high concentrations of $PDGF_{AA}$ and HGF. Similarly, there were high concentrations of VEGF and $PDGF_{BB}$ in HeLa cell culture supernatants, and these results are in accordance with the findings reported by Zhang et al. (2014), in which the authors found that high concentrations of HGF and $PDGF_{AA}$ were related to the development of HPV^+ cervical carcinoma. Differences in the concentrations of growth factors in HeLa and SiHa cell culture supernatants could be caused by the HPV E6 and E7 oncoproteins and their direct role in the stimulation of angiogenesis mediators [22,23]. On the other hand, VEGF induces cell proliferation and vascular permeability, thus promoting tumor development. A high expression of VEGF has been reported in CxCa; however, we observed that VEGF was differentially produced by the three CxCa cell lines, with HeLa cells producing the highest amount. The latter can be explained by the fact that human telomere reverse transcriptase is upregulated by the HPV-18 E7 oncoprotein, which upregulates VEGF expression [24,25].

From our analysis of members of the TGF-β protein family, we only detected TGF-β3 in HeLa, SiHa, and C-33A cell culture supernatants. This result corresponds with those of a previous study, in which it was observed that TGF-β expression decreased with an increasing severity of dysplasia in CxCa samples [26]. In particular, El-Sherif et al. (2000) observed a decrease of TGF-β expression in $HPV\text{-}16^+$ cervical precancerous lesion samples, which could explain why SiHa secreted the lowest amounts of TGF-β3 [27–29].

We found that $PDGF_{AA}$, VEGF, HGF, and TGF-β3 are produced by CxCa cells, and these growth factors have been described as possessing a dual role in several cancers. First, these factors promote angiogenesis, metastasis, cell recruitment, and tumor growth [30,31]. Second, they induce an M2-like phenotype in macrophages, which has important functions in maintaining an immunosuppressive microenvironment and promoting angiogenesis and metastasis [32].

TGF-β3 was the only secreted factor to be detected in the culture media of all experimental groups of THP-1 macrophages stimulated with the CxCa cell culture supernatants. TGF-β3 expression plays an important role in developmental biology, while in melanoma and breast cancer, its high expression is related to an increased severity of disease, metastasis, and a poor prognosis [33]. However, the roles of TGF-β3 in cervical cancer and immune cells remain unclear. Therefore, more studies are necessary to understand the role of TGF-β3 in the tumor microenvironment.

In our study THP-1 macrophages treated with CxCa cell culture supernatants released large amounts of PDGF, suggesting that the macrophages acquire tumor-promoting activities. It has been described that macrophages of M2-like phenotypes secrete distinct growth factors, such as PDGF and ANG-2, that promote tumor growth, angiogenesis, tissue remodeling, and repair [34–36].

The cytokine IL-6 is an important element in the induction of M2-like macrophages. This cytokine has been described as participating in monocyte differentiation into tumor-associated macrophage-like cells alongside autocrine/paracrine M-CSF signaling [37]. THP-1 macrophages stimulated with CxCa cell culture supernatants in our study secreted increased levels of IL-6 and decreased levels of M-CSF, and these changes could perhaps be related to the autocrine induction of the M2 phenotypic switch (Fig. 4B and C). However, this is not the only pathway through which IL-6 polarizes macrophages towards the M2 phenotype, since IL-6 can also activate the JAK/STAT3 pathway, which induces the expression of hypoxia inducible factor 1α and VEGF [38]. This alternative pathway of M2 phenotype induction by IL-6 is particularly interesting in the context of this study, because we observed a high production of VEGF and IL-6 in the THP-1 macrophages, especially in those treated with the HeLa cell culture supernatant.

CD206 expression is regulated by IL-4 and IL-13 in M2 macrophages with immunosuppressive activities [39–41]. Although we did not observe a clear increase of CD206 expression in THP-1 macrophages treated with CxCa cell culture supernatants in this study, this could be due to the fact that we did not detect high concentrations of IL-4 in the CxCa cell culture supernatants.

When we analyzed receptors, cytokines, growth factors, and transcription factors related to the M1 and M2 macrophage phenotypes, the induction of an M2-like phenotype in THP-1 macrophages treated with CxCa cell culture supernatants was indicated by an increased expression of several M2 phenotype markers (see Table 2). The induction of the M1 phenotype in macrophages is associated with a high expression of STAT1 [42,43]. In this study, we observed that pSTAT1 expression was only induced in THP-1 macrophages stimulated with IFN-γ plus LPS, while THP-1 macrophages treated with CxCa cell culture supernatants exhibited a decreased expression of pSTAT1 and an unchanged expression of pSTAT6. Other signaling pathways could be involved in the induction of an M2-like phenotype in the THP-1 macrophages, including the PI3K, STAT3, and IRF4 pathways [43]. However, more studies are necessary to determine the precise mechanisms underlying this phenotypic switch.

Interestingly, when we removed the CxCa cell culture supernatants from THP-1 macrophages and incubated the macrophages for a further 48 h, these cells maintained their production of $PDGF_{AA}$, $PDGF_{BB}$, and VEGF to a similar or lesser degree, while their release of NO was diminished. These findings suggest that several

Table 2
Comparative analysis of the expression of soluble factors, membrane receptors, and transcription factors by THP-1 macrophages treated with CxCa supernatants vs. M1 and M2 macrophages. The expression of cytokines, growth factors, membrane receptors, and transcription factors in THP-1 macrophages treated with HeLa, SiHa, and C-33A cell culture supernatants was compared against the corresponding expression profiles of M1 and M2 macrophages. The control groups were Mθ macrophages (baseline phenotype), M1 macrophages (treated with IFN-γ + LPS), and M2 macrophages (treated with IL-4 + IL-10). sHeLa, sSiHa, and sC-33A represent the supernatants of three different CxCa cell lines. ↑: increased expression; ↓: decreased expression; and -: equal expression.

		MΘ+sHeLa	MΘ+sSiHa	MΘ+sC-33A
M1	GM-CSF	↓	↓	↓
	IFNγ	↓	↓	↓
	NO	↓	↓	↓
	TNFα	↓	↓	↓
	CD80	-	↓	↑
	CD86	-	-	-
	HLA-DR	↓	↓	↑
M2	CD206	↓	-	↑
	CD163	↑	-	↑
	IL-4	↑	↑	-
	HGF	↑	↑	↑
	PDGF-AA	↑	↑	-
	VEGF	↑	-	-

factors secreted by CxCa cell lines disrupt signaling pathways in macrophages and this effect could promote proliferation, angiogenesis, and metastasis in cervical cancer.

5. Conclusions

HeLa, SiHa, and C-33A cancer cells secrete diverse factors involved in tumor growth, such as IL-6, $PDGF_{AA}$, $PDGF_{BB}$, HGF, VEGF, and ANG-2, which induce the polarization of THP-1 macrophages towards an M2-like phenotype. Interestingly, the macrophages maintained their secretion of $PDGF_{AA}$, $PDGF_{BB}$, and VEGF for at least 48 h after the CxCa cell culture supernatants were removed. Macrophages of the M2 phenotype are thought to promote tumor growth and are also a source of a feedback loop by inducing the M2 polarization of other macrophages.

Competing interests

The authors declare no potential conflicts of interest.

Authors' contributions

Pablo Cesar Ortiz-Lazareno, Eliza J. Pedraza-Brindis, Alejandro Bravo-Cuellar, and Georgina Hernández-Flores designed and performed the research, analyzed the data, and drafted the paper. Paulina Gómez-Lomelí, Brenda A. López-López, Adriana Aguilar-Lemarroy, Luis Felipe Jave-Suárez, and Karina Sánchez-Reyes performed the measurements of cytokines and growth factors, analyzed the data, and drafted the paper.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.cellimm.2016.07.001.

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