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Human Papillomavirus E6 and E7 oncoproteins affect the cell microenvironment by classical secretion and extracellular vesicles delivery of inflammatory mediators

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ABSTRACT

The connection between chronic inflammation and risk of cancer has been supported by several studies. The development of cancer might be a process driven by the presence of a specific combination of inflammatory mediators, including cytokines, chemokines and enzymes, in the tumor microenvironment. Virus-induced tumors, like HPV-induced Squamous Cell Carcinomas, represent a paradigmatic example of the interplay between inflammation, as integral part of the innate antiviral response, and malignant transformation. Here, the role of inflammatory microenvironment in the HPV-induced carcinogenesis is addressed, with a specific focus on the involvement of the immune molecules as well as their delivery through the microvesicle cargo possibly correlated to the different HPV genotype.

The expression of the inflammatory mediators in HPV positive cells has been analyzed in primary human foreskin keratinocytes and keratinocytes transduced by E6 and E7 from mucosal HPV-16 or cutaneous HPV-38 genotypes. HPV E6 and E7 proteins can modulate the expression of immune mediators in HPV-infected cells and can affect the levels of immune molecules, mainly chemokines, in the extracellular milieu. HPV-16 E6 and E7 oncoproteins have been silenced to confirm the specificity of the modulation of the inflammatory microenvironment.

Our results suggest that the expression of HPV oncoproteins allows the modification of the tumor milieu through the synthesis and release of specific pro-inflammatory cytokines and chemokines, affecting the efficacy of the immune response. The microenvironment can also be conditioned by an altered mRNA cargo delivered by extracellular vesicles, thereby efficiently affecting the surrounding cells with possible implication for tumorigenesis and tumor diagnosis.

1. Introduction

The inflammatory microenvironment, established by inflammatory cells, cytokines, chemokines and enzymes, plays a fundamental role in the development of tumor [1]. Indeed, people inclined to chronic inflammatory diseases have a higher risk to develop tumors [2].

Cancer secretome represents the whole proteins released by transformed cells or tissues (cytokines, hormones, coagulation and growth factors) that possibly participate to several physiological and pathological processes. Moreover, the interplay among cells or cell-to-extracellular matrix is another fundamental process of carcinogenesis [3].

Some chemokines (CXCL1/GRO-α, CXCL5/ENA-78, CXCL8/IL-8, CXCL12/SDF-1) and pro-inflammatory cytokines as interleukin (IL)-1α and IL-6 have the potentiality to support tumor growth otherwise the treatment with non-steroidal anti-inflammatory drugs has effects on the reduction of cancer incidence and aggressiveness [4]. On the other hand, toll like receptors (TLRs) can induce both anti- and pro-tumorigenic pathways thus affecting the tumor microenvironment [5]. Various members of the Toll Like Receptor (TLR) family can be inhibited by

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some viruses like Epstein-Barr Virus (EBV), Hepatitis B virus (HBV) and mucosal Human Papillomavirus (HPV) type 16 that reduce TLR9 expression and function [6–8]. About 15% of the global cancer burden is attributable to infectious agents and a persistent inflammation characterizes these chronic infections; for example, hepatocellular, colorectal, cervical and gastric carcinomas share a chronic inflammatory response [1]. Oncogenic viruses, such as Hepatitis C virus (HCV), HPV and HBV, produce persistent infection in which the viral oncoproteins alter specific cellular pathways promoting tumorigenesis. A continuous and deregulated immune response produces serious systemic and tissue damage, impaired tumor surveillance and, finally, cancer characterized by metastatic phenotype and resistance to chemotherapy [9].

HPVs are a large family of small DNA viruses that can be classified by their tropism to skin or mucosa [10]. High risk (HR)-HPVs belong to mucosal genotypes and are the aetiological agent of cervical cancer whereas the involvement of specific cutaneous HPV types in human carcinogenesis is not completely demonstrated. High risk genotypes exert their dangerous functions mainly through E6 and E7 oncoproteins that are already highly expressed in precursor lesions where they interact with numerous cellular proteins finally fostering viral immune-evasion and tumor development. About 60% of all cervical cancers are caused by mucosal HPV-16, as well as some anogenital and head and neck cancers. The properties of most cutaneous HPV types are unknown but it has been reported that E6 and E7 proteins of some cutaneous HPVs, such as HPV-38, have transforming properties [11].

In contrast to the increasing knowledge of the intracellular activities of E6 and E7 oncogenes, the possible effects on the intercellular communication of HPV-positive cancer cells is not yet well known.

The production and release of extracellular vesicles (EVs) like microvesicle, which blebs directly from plasmamembrane and exosomes, small extracellular vesicles of endocytic origin that can contain proteins, RNA, microRNAs and DNA [12], is deregulated in cancer, indicating their important role in tumors [13–15]. The release of EVs from cancer cells can impair the microenvironment, affecting tumor development and chemoresistance [16,17]. Rising evidence suggests that cancer cells use EVs transmitted nucleic acids and proteins to evade an immune response [18].

Tumor viruses can lead to modifications of the content of EVs ultimately implying them into the intercellular communication of virally transformed cells. Novel diagnostic markers for virus-associated preneoplasia or neoplasia could be found from the characterization of new virus-induced molecular signatures, as, for example, EVs.

Here we report that the inflammatory microenvironment appears modified in the HPV-induced transformation. In particular HPV E6 and E7 proteins can modulate the expression of immune mediators in HPV-infected cells and can affect the levels of immune molecules, mainly chemokines, in the extracellular milieu. In addition, the inflammatory immune mediators delivery through the EVs is affected by the expression of the HPV oncoproteins.

2. Materials and Methods

2.1. Cell cultures and treatments

Primary Human Foreskin Keratinocytes (HFK) were transduced with pLXSN16E6E7 (K16) and pLXSN38E6E7 (K38) as already described [19] and were grown in serum free keratinocytes medium (KBM BulletKit, Lonza). HFK were utilized as control. SiHa cell line was grown in DMEM + 10% fetal calf serum. Cells were cultivated in a humidified air of 5.0% CO_2 at 37 °C.

2.2. Extracellular vesicles isolation

HFK, K16 and K38 cells were seeded at 3×10^6 cells/plate in 100 mm tissue culture plates. After 5 days supernatants were collected and centrifuged at 500*g* for 10 min, 2000*g* for 10 min and 100000*g* for 60 min. At the end of the last spin, supernatants were discarded and the pellet containing the EVs was stored at −80 °C for the subsequent analyses.

2.3. HPV-16 E6, HPV-16 E7 silencing

Small interfering RNAs (siRNAs) conjugated with phosphoramidite (FAM) and targeted to HPV-16 E6 and HPV-16 E7 were designed and approved by Qiagen and a nonsilencing siRNA (Qiagen) was used as control.

HPV-16 E6 siRNAs were:
sense GAGGUAUAUGACUUUGCUU;
antisense AAGCAAAGUCAUAUACCUC.
HPV-16 E7 siRNAs were:
sense AGGAGGAUGAAAUAGAUGG;
antisense CCAUCUAUUUCAUCCUCCU.
Control siRNAs were:
sense UUCUCCGAACGUGUCACGU;
antisense ACGUGACACGUUCGGAGAA.

Briefly, before transfection 2×10^5 cells were seeded in 35-mm dishes in 1 ml of supplemented culture medium. siRNA was diluted in 50 µl of culture medium up to 10 nM final concentration. Successively 3.5 µl of HiPerfect Transfection Reagent (Qiagen) was added to siRNA-medium solution and mixed by vortexing. After 10 min at room temperature, the transfection complex was included drop-wise onto the cells. Twenty-four hours after transfection, transfection efficiency was evaluated by cytometric analysis (FACS Aria II as instrument and FACS Diva 6.1.1 as software, both from Becton–Dickinson). At least 10^4 events were acquired and analyzed using Flowing software (version 2.5.1, Turku Centre for Biotechnology). The level of transfected cells typically was superior to 50%.

2.4. RNA purification and Real Time RT-PCR

Cells were seeded in six well plates, 2×10^5 cell/ml and, after 72 h, were washed twice in PBS. EVs were isolated as described above. Cellular or EVs-derived RNA was purified utilizing the Total RNA Purification Kit (Norgen Biotech Corp.). Five hundred ng to 1 µg of total RNA were retro-transcribed using the Tetro cDNA synthesis kit (Bioline) and cDNA were examined by real-time PCR using the Sensi-Mix SYBR Hi-ROX Kit (Bioline) and specific primers (Table 1).

2.5. Western blot

K16 cells were seeded at 3×10^6 cells/plate in 100 mm tissue culture plates and 2 h later cells were transfected with HPV-16 E6 and E7 siRNAs or control siRNA. Following 72 h cells were tripsinized and pellets were gathered. Cells were lysed in lysis buffer (20 mM Tris-HCl pH 8, 200 mM NaCl, 0.5% Nonidet P-40, 1 mM EDTA, 10 mM NaF, 0.1 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride, 1 µg/ml leupeptin, 1 µg/ml aprotinin) and 30 µg of total proteins were resuspended in 5X SDS sample buffer (40% Glycerol, 240 mM Tris–HCl pH 6.8, 8% SDS, 0.04% bromophenol blue, 5% beta-mercaptoethanol), resolved on SDS–PAGE and transferred onto nitrocellulose membrane (Whatman). The membranes were incubated with mouse anti-p53 (clone Bp53-12 Santa Cruz) and rabbit monoclonal anti-GAPDH (clone 14C10, Cell Signaling). Immune complexes were detected with horseradish peroxidase-conjugated rabbit anti-mouse and goat anti-rabbit (Calbiochem).

2.6. Human cytokine Antibody Array – Membrane

K16 and K38 cells were seeded in six well plates (2×10^5 cells/ml). After 72 h supernatants were collected and centrifuged at 2000*g* for 10 min. Subsequently, Human Cytokine Antibody Array – Membrane encompassing 80 cytokines, chemokines, growth and angiogenic factors (Abcam) was performed according to the manufacturer's instructions.

Table 1
Primers used in Real Time RT-PCR.

Gene	Forward 5′ - 3′	Reverse 5′ - 3′
IL-1α	CGCCAATGACTCAGAGGAAGA	AGGGCGTCATTCAGGATGAA
IL-1β	GCTTATTACAGTGGCAATGAGGAT	GGTGGTCGGAGATTCGTAG
IL-6	GGTACATCCTCGACGGCATCT	GTGCCTCTTTGCTGCTTTCAC
CCL2/MCP-1	CCCCAGTCACCTGCTGTTAT	TGGAATCCTGAACCCACTTC
CCL20/MIP-3α	TGGCCAATGAAGGCTGTGA	GATTTGCGCACACAGACAACTT
CCL27/CTACK	AGCTCTACCGAAAGCCACTC	GAGCCAGGTGAAGCACGAA
CXCL8/IL-8	CTTGGCAGCCTTCCTGATTT	TTCTTTAGCACTCCTTGGCAAAA
CXCL1/GRO-α	CCAAACCGAAGTCATAGCCA	CTTCCTCCTCCCTTCTGGTC
CXCL3/GRO-γ	ATCCCCCATGGTTCAGAAA	ACCCTGCAGGAAGTGTCAAT
CXCL10/IP-10	CACACTAGCCCCACGTTTTCT	TGGTGCTGAGACTGGAGGTT
Angiogenin	TCCATTGTCCTGCCCGTTTC	GCCCTGTGGTTTGGCATCAT
TNF-α	ATCTTCTCGAACCCCGAGTGA	CGGTTCAGCCACTGGAGCT
CCL5/RANTES	TCTGCGCTCCTGCATCTG	GGGCAATGTAGGCAAAGCA
HPV-16 E6	AGCGACCCAGAAAGTTACCA	GCATAAATCCCGAAAAGCAA
HPV-16 E7	ACAAGCAGAACCGGACAGAG	GCCCATTAACAGGTCTTCCA
HPRT-1	AATTATGGACAGGACTGAACGTCTTGCT	TCCAGCAGGTCAGCAAAGAATTTATAGC
18S	GCAATTATTCCCCATGAACG	GGGACTTAATCAACGCAAGC

2.7. Statistical analysis

Statistical analysis were performed using Prism Software (v6.0c, GraphPad Software Inc.). Depending on the experiment, data were analyzed using One-way ANOVA followed by Kruskal-Wallis test, or *t*-test followed by Mann-Whitney post test. ∗ P < .05; ∗∗ P < .01; ∗∗∗ P < .001; ∗∗∗∗ P < .0001.

3. Results

3.1. The expression of cytokines and chemokines is modulated in K16 and K38 cells

In order to analyze the expression of inflammatory mediators in keratinocytes expressing E6 and E7 of mucosal and cutaneous HPV, Real Time RT-PCR experiments were performed on mRNA of K16 and K38 cells compared to mRNA of HFK. Among the examined cytokines and chemokines, IL-1α, -1β, -6, CCL2/MCP-1, CCL20/MIP-3α, CCL27/CTACK, CXCL8/IL-8, CXCL1/Gro-α and CXCL10/IP-10 are all downregulated in both K16 and K38 cells. On the other hand, CXCL3/Gro-γ and Angiogenin were downregulated in a statistical significant manner only in K38 cells whereas TNF-α and CCL5/RANTES were upregulated only in K16 (Fig. 1).

3.2. HPV-16 E6 and E7 silencing counteracts the modulation of the inflammatory mediator expression in K16 cells

The involvement of HPV-16 E6 and E7 oncoproteins in the expression of the inflammatory mediators was investigated in K16 cells. For this purpose, keratinocytes were silenced for E6 and E7 expression by using specific FAM-conjugated siRNAs. High levels of siRNA expression were recorded by flow cytometry in K16-transfected cells (Fig. 2A). As expected, E6 and E7 silencing in K16 cells inhibits the expression of E6 and E7 mRNAs (Fig. 2B, C) and restores the expression of p53 (Fig. 2D) compared to K16 cells transfected with a scramble siRNA.

Real Time RT-PCR experiments show that E6 and E7 silencing counteracts the effect exerted by K16 cells on the expression levels of cytokines and chemokines, indicating that the HPV oncoproteins are involved in the modulation of the inflammatory mediator expression (Fig. 2E). These results were confirmed also in experiments performed in the HPV-16 positive SiHa cell line (data not shown).

3.3. Supernantants from K16 and K38 cells display a specific pattern of secreted cytokines and chemokines

To test if the deregulation of cyto/chemokine gene expression exerted by E6 and E7 goes with a parallel altered secretion, supernatants collected from HFK, K16 and K38 cells were analyzed by Human Chemokine Antibody Array-Membrane (Abcam). According to gene expression, Gro, CXCL1/Gro-α and CXCL8/IL-8 are downregulated in both the supernatants of K16 and K38 compared to HFK. In addition, Angiogenin, CXCL10/IP-10, CCL5/RANTES and TIMP-1 are upregulated only in K16, while CCL2/MCP-1 is upregulated in K38 supernatant (Fig. 3). These results were confirmed on E6/E7 silenced K16 cells using the human common chemokines multi-analyte ELISArray kit (Qiagen, data not shown).

3.4. HPV oncoproteins affect the inflammatory mediators mRNA cargo in EVs derived from E6 and E7 expressing keratinocytes

Since we reported the ability of HPV oncogenes to deregulate the expression of several pro-inflammatory cyto- and chemokines, we wondered if a similar modulation takes place also in EV-embedded mRNAs in our cellular model. To this purpose, EVs released from HFK, K16 and K38 were collected and pro-inflammatory cyto/chemokines mRNAs quantified by Real Time RT-PCR. As reported in Fig. 4, K16- and K38-derived EVs do not contain CCL-27/CTACK and CXCL3/Gro-γ. Angiogenin and CCL20/MIP-3α mRNAs are absent only in K16-derived EVs, whereas CXCL8/IL-8 is present and equally expressed in EVs from K16 and K38 compared to EVs from HFK. According to the data obtained into the parental cell lines, IL-1α, -1β and CXCL1/Gro-α mRNAs are all downregulated in EVs derived from K16. On the other hand, CXCL10/IP-10 in K16cells and CCL2/MCP-1 in K38 cells are over-represented in EVs in a statistically significant manner.

4. Discussion

Several methods to avoid recognition, dendritic cell (DCs) and macrophage activation and by consequence the induction of both the innate and the cell mediated immune response have been excogitated by most viruses. In the case of oncogenic viruses, these functions were further implemented with the capacity to tune the release of cytokines, chemokines, growth factors and altered EVs leading to the formation of settings characterized by an immunosuppressive and/or an immunomodulatory microenvironment. HPVs avoid viral antigens presentation to immune and epithelial cells. Keratinocytes of the basal layer are infected without damages of the basal lamina [20], the viral replication cycle is linked to the keratinocytes terminal differentiation [21] and viral assembly and release occur concurrently with the programmed cell death of these cells. Collectively, those features minimize the release of inflammatory mediators and danger signals able to evoke the immune response by skin resident immune cells [22].

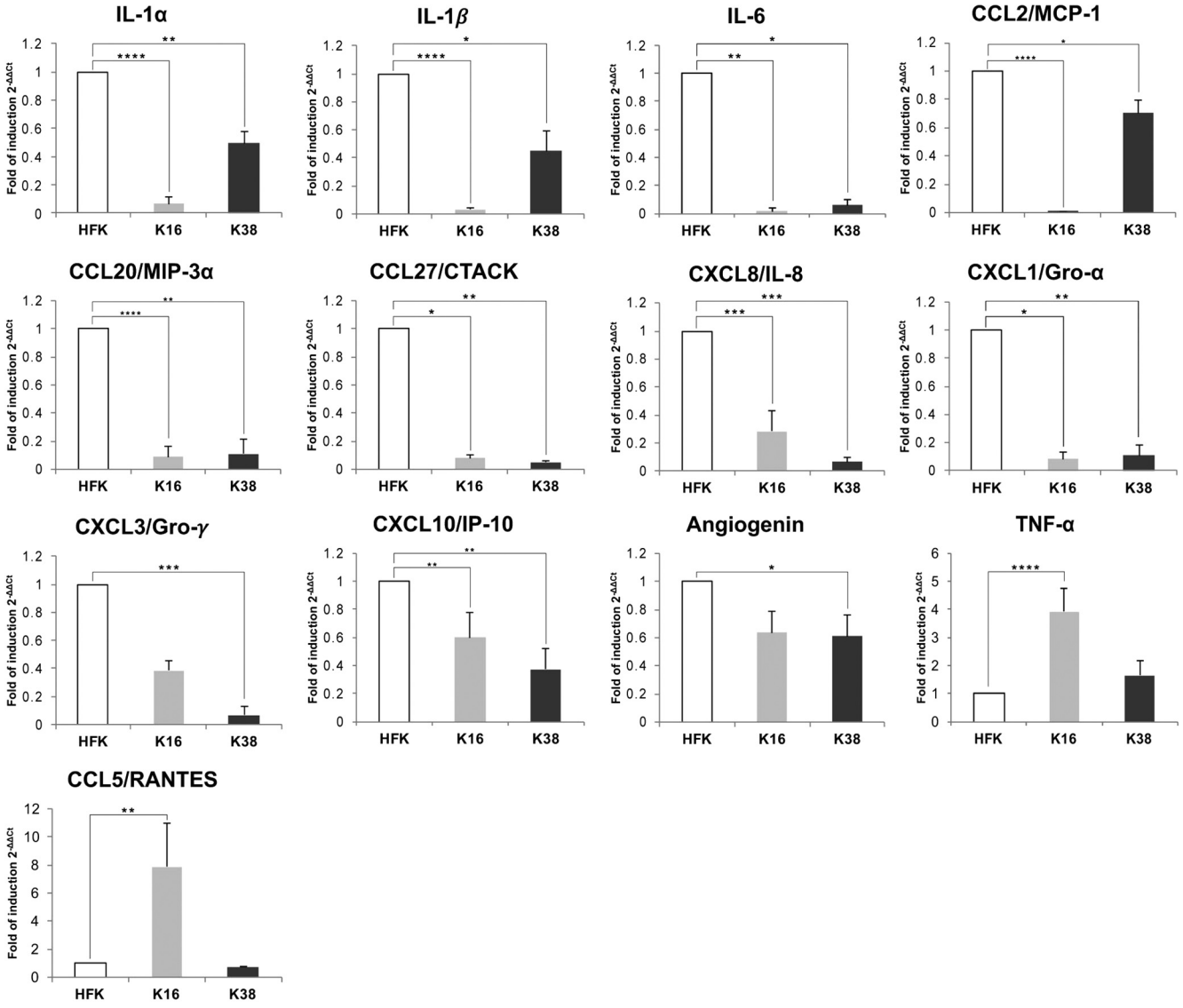

Fig. 1. Expression levels of inflammatory cytokines and chemokines in K16 and K38 cells. Total RNA was extracted by using Total RNA purification Kit (Norgen Biotech Corp.) from HFK, K16 and K38 cells after 72 h from seeding. Sybr Green-based Real Time RT-PCR was performed for each retro-transcribed sample by using specific primers. Results were expressed as fold of induction ($2^{-\Delta\Delta CT}$ method) using HPRT-1 as an internal loading control and HFK cells as reference. Data represent means ± s.d. of three independent experiments. Statistical analysis was performed by using one-way ANOVA following by Kruskal-Wallis test. * P < .05; ** P < .01; *** P < .001; **** P < .0001.

Genetic rearrangements, activation of proto-oncogenes and other events able to determine genomic instability are necessary but not sufficient requirements for the neoplastic transformation induced by HPV infection even when viral DNA integration into the host cell genome occurs. Beside their activities on p53 and pRB, respectively, E6 and E7 also repress at different levels the NF-κB signaling. Whilst E7 attenuates NF-κB activation by targeting the IKK complex, E6 induces the cytoplasmic retention of p100 and p105 NF-κB subunits thereby impeding nuclear translocation and binding to promoter sequences [23–25]. This evidence suggests that NF-κB suppression mediated by viral oncogenes plays a pivotal role in the escape from immune recognition and transformation. In our experiments, the expression of inflammatory cytokines is modulated in cells expressing E6 and E7 proteins of HPV-16 and HPV-38. In particular, IL-1α, -1β, -6, are all downregulated in both K16 and K38 cells, whereas TNF-α appears upregulated (Fig. 1). Silencing experiments indicate that the HPV oncoproteins are involved in the modulation of the inflammatory mediator expression (Fig. 2). The discrepant induction of TNF-α mRNA compared to the other pro-inflammatory cytokines might be explained by a possible NF-κB-independent induction of this cytokine as already reported in activated T cells [26]. In the case of HPV the upregulation of TNF-α might be correlated to the ability of HPV to activate Erk MAPKs pathway [27].

Together with the capacity to down-modulate proinflammatory cytokines and to skew cytokine production toward an immunosuppressive/Th2 phenotype, HPV is also able to alter the production of chemokines in keratinocytes thereby perturbing leukocytes trafficking into the skin. From this point of view CCL20/MIP-3α represents the main target. It has been demonstrated that CCL20/MIP-3α is the main chemotactic factor for Langerhans cell (LCs) and DCs [28]. In epithelial lesions expressing HPV-16, CCL20/MIP-3α is inversely correlated to E6 and E7 whereas it is directly correlated to LCs infiltrate [29]. Further, CCL20/MIP-3α production and LCs chemotaxis is restored after E6 and E7 silencing [30]. Notably, non HR-HPVs are also enabled to repress CCL20/MIP-3α. Indeed, it has been reported that in HPV-8$^+$ lesions from epidermodysplasia verruciformis patients, the expression of this chemokine is reduced as well as the LCs infiltrate [31]. CCL20/MIP-3α plays also a pivotal role in the trafficking of Th17 lymphocytes into inflammatory skin disease as psoriasis [32]. This observation reinforces the idea that HPV operates to dampen

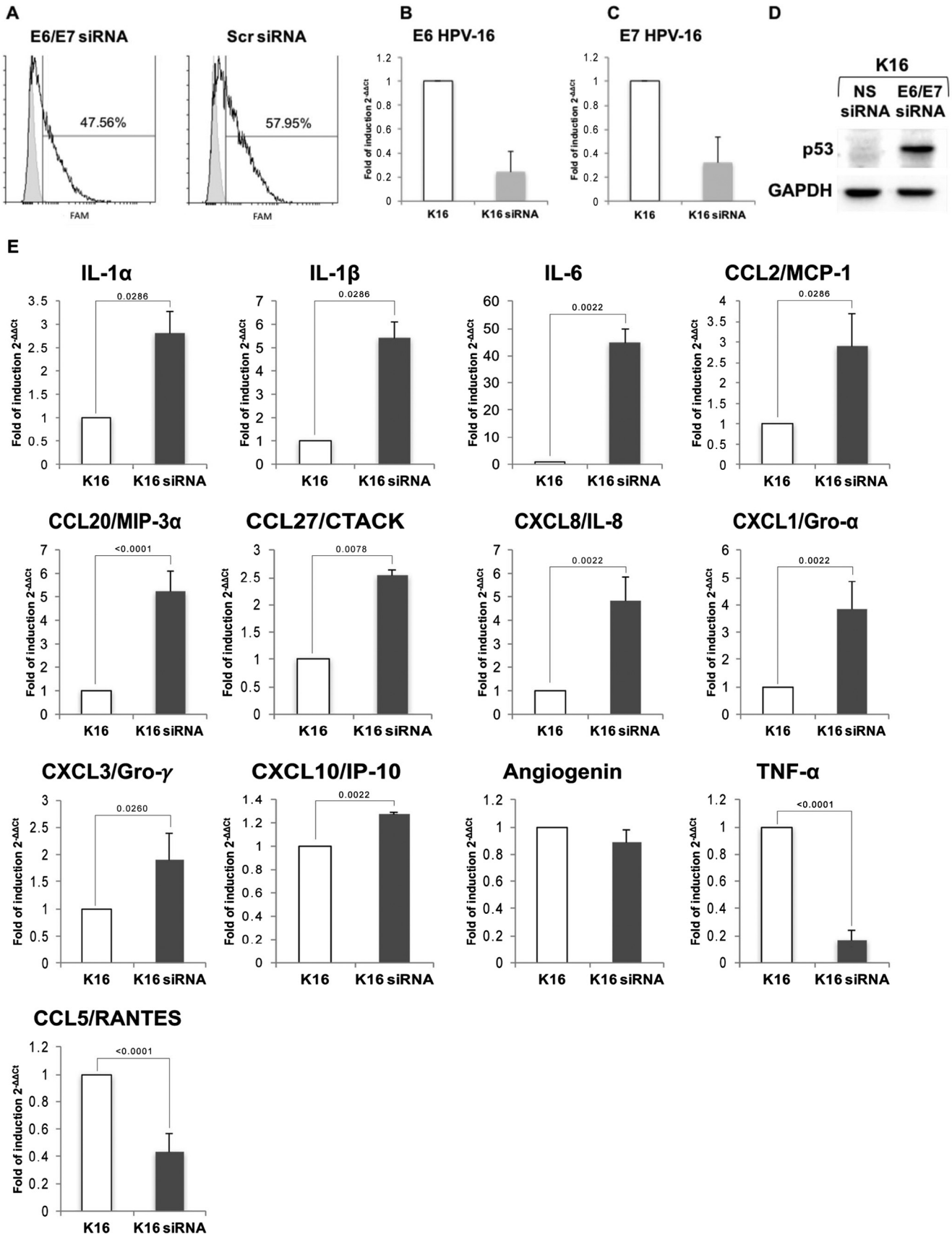

Fig. 2. Expression levels of inflammatory cytochines and chemokines in K16 and K16 E6/E7 silenced cells. K16 cells were seeded in six well plates (2×10^5 cells/ml) and after 2 h cells were transfected with E6 and E7 FAM-siRNA or scramble siRNA (10 nM) using HiPerFect transfection reagent (Qiagen). After 24 h the transfection efficiency was evaluated by flow cytometry (A). After 72 h cells were washed twice in PBS and whole cell extracts were analyzed by SDS-PAGE western blot of p53, as reported in "Materials and Methods" (D). Moreover total RNA was extracted, retro-transcribed and Real Time RT-PCR performed to evaluate K16 silencing for E6 (B) and E7 (C) and the expression levels of cytokines and chemokines showed in Fig. 1 (E). Data represent means ± s.d. of three independent experiments. Statistical analysis was performed by using *t*-test following by Mann-Whitney post test. Exact P value was indicated where $P < .05$.

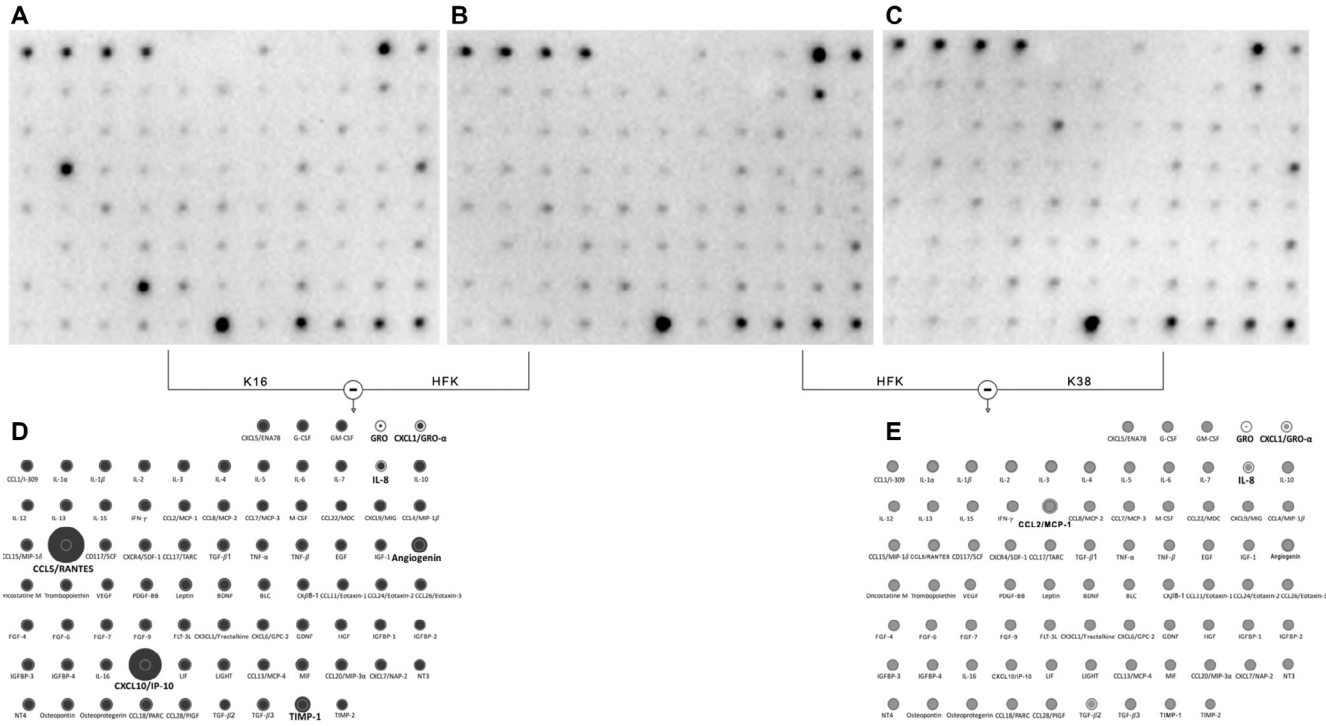

Fig. 3. Cytokines and Chemokines detection in supernatants collected from HFK, K16 and K38. Cells were seeded in six well plates (2×10^5 cells/ml). After 72 h supernatants were collected and centrifuged 2000*g* for 10 min. Subsequently Human Cytokine Antibody Array - Membrane (Abcam) was performed according to the manufacturer's instructions. Data were schematized comparing HFK membrane (B) to K16 (A) or K38 (C). Briefly, rings represent the amount of the inflammatory mediators secerned by HFK while disks are the quantity derived from K16 (D) or K38 (E) supernatants analyses. A down-modulation of a specific mediator shows a disk smaller than the ring, otherwise a bigger disk represents an up-modulation.

proinflammatory responses. Our data have not only confirmed the above reported results in HPV-positive cells, at least at mRNA level (see Figs. 1 and 2), but have also extended these observations to the cargo of EVs released from HPV-positive keratinocytes in which CCL20/MIP-3α mRNA is virtually absent (Fig. 3).

HPV-16 E6 and E7 suppress the expression of CCL2/MCP-1 in cervical epithelial as well as in epidermal cells even if the mechanism used by the oncoproteins to downregulate this chemokine is still missing [33]. On the other hand, Tumor Associated Macrophages (TAM) release CCL2/MCP-1 in the tumor microenvironment thereby inducing the recruitment of monocytes which are polarized into M2/like pro-tumoral cells [34]. HPV also downregulates the expression of CXCL8/IL-8 [35].

Secreted factors released by cancer cells strongly affect cancer progression. Differentiation, invasion, metastasis and angiogenesis of cancer are regulated by cytokines, chemokines, proteases as well as growth and angiogenic factors through their ability to modulate cell-to-cell and cell-to-matrix interactions. Those factors could be retrieved and measured in different body fluids (i.e. blood, urine). Thus the "cancer secretome" can provide new tools to clarify the molecular mechanisms of carcinogenesis leading to the discovery of novel biomarkers [3]. Our results indicate that the expression of soluble factors other than cytokines and chemokines as Angiogenin and TIMP-1 is modulated in cells expressing E6 and E7 proteins of HPV-16 and HPV-38. In particular, Angiogenin is downregulated in both K16 and K38 at mRNA level but its secretion is slightly enhanced in the supernatants of K16 (Figs. 1 and 3). TIMP-1 secretion is also upregulated only in supernatants from K16 cells (Fig. 3). This scenario might be compatible with increased pro-angiogenic and pro-metastatic activities modulated by E6 and E7 oncoproteins.

Protein detection experiments in the supernatants of K16 and K38 cells compared to HFK, performed to follow the canonical cytokine secretion, show that Gro, CXCL1/Gro-α and CXCL8/IL-8 are downregulated, while Angiogenin, as already mentioned, is slightly upregulated in both K16 and K38 supernantants. CXCL10/IP-10 and CCL5/RANTES are upregulated only in K16, while CCL2/MCP-1 is upregulated in K38 supernatant (Fig. 3A and B).

Tumors are able to bias the microenvironment not only through the modulation of cytokines and chemokines but also through the control of release and cargo content of EVs [36]. To date, it has been reported the ability of EVs isolated from HPV positive cells to deliver microRNAs and cellular proteins to microenvironment and acceptor cells [37–41]. The analysis performed on the mRNA cargo of EVs released by K16 and K38 essentially recapitulates the scenario observed in the parental cells with some minor differences (see Fig. 1 compared to Fig. 4), indicating the ability of the HPV oncoproteins to affect not only microRNA expression but also mRNA content in EVs. These differences, as for example the higher content of CCL2/MCP-1 and CXCL10/IP-10, could be due to the presence in these mRNAs of specific signal sequences able to target them for exosome and/or microvesicle inclusion.

5. Conclusions

Data shown here indicate a role of E6 and E7, from both mucosal high-risk and cutaneous HPVs, in the down-modulation of a series of inflammatory cyto- and chemokines. In particular, HPV E6 and E7 alter the extracellular milieu in a cancer microenvironment characterized by immunosuppression, evasion and gene expression control in surrounding non-transformed cells.

Furthermore, E6/E7-dependent changes in the content of extracellular vesicles have a role in the deregulation of the extracellular microenvironment as well as in the regulation of cellular functions in non-transformed acceptor cells and/or immune surrounding cells, through the transfer of EV content.

Conflict of interest

The authors declare that they have no conflict of interest.

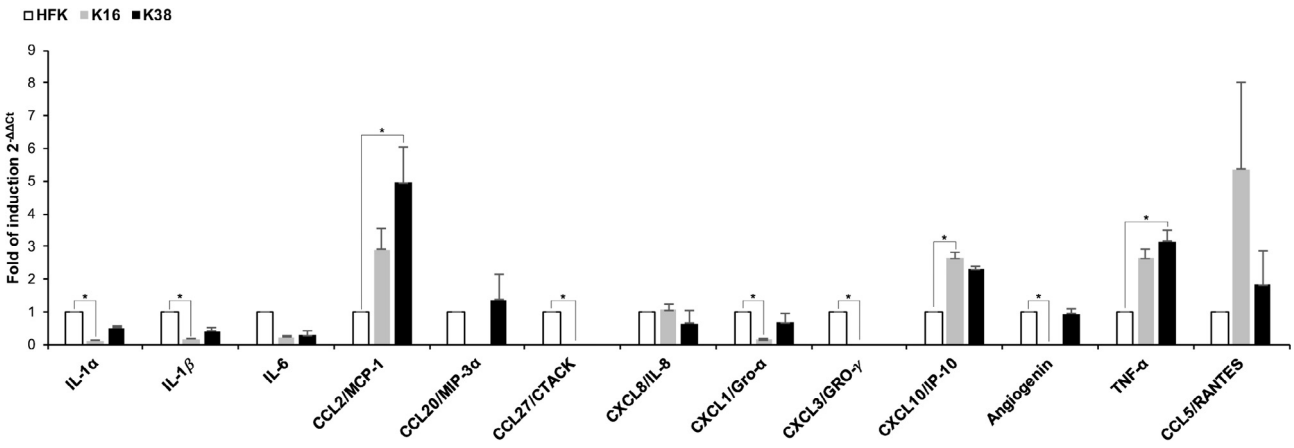

Fig. 4. Expression levels of cytokines and chemokines related to inflammation in K16 and K38 derived EVs. Total RNA was extracted by using Total RNA purification Kit (Norgen Biotech Corp.) from HFK, K16 and K38 derived EVs 5 days after seeding. EVs were purified as described in "Materials and Methods". Sybr Green-based Real Time RT-PCR was performed for each retro-transcribed sample by using specific primers. Results were expressed as fold of induction ($2^{-\Delta\Delta CT}$ method) using 18S as an internal loading control and HFK cells as reference. Data represent means ± s.d. of three independent experiments. Statistical analysis was performed by using one-way ANOVA following by Kruskal-Wallis test. * $P < .05$.

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