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## SELECTIVE SUPPRESSION OF MONOCYTE CHEMOATTRACTANT PROTEIN-1 EXPRESSION BY HUMAN PAPILLOMAVIRUS E6 AND E7 ONCOPROTEINS IN HUMAN CERVICAL EPITHELIAL AND EPIDERMAL CELLS

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**Infection of cervical keratinocytes by high-risk HPV is involved in the etiology of cervical carcinoma. Since viral products are immunogenic, development of cancer may require suppression of immune responses directed against infected epithelial cells. Many markers of host immune effector responses decrease as cervical intraepithelial neoplasia progresses. Among these is epithelial cell expression of the chemokine MCP-1, though the mechanism for its suppression is unclear. Here, we show that the E6 and E7 viral oncogenes from high-risk HPV, individually and together, suppress MCP-1 expression in primary epithelial cells derived from the female genital tract. This is not a consequence of global suppression of chemokine expression since other chemokines, including IP-10, IL-8 and RANTES, were less affected. Furthermore, 4 of 6 HPV-positive cervical carcinoma cell lines did not express MCP-1. Our data indicate that suppression of MCP-1 expression is part of the program of high-risk HPV E6/E7-induced transformation of primary epithelial cells. These observations are consistent with a model in which MCP-1 expression by infected keratinocytes, which would stimulate an immune attack on HPV-transformed cells, is suppressed for invasive cervical cancer to appear.**

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**Key words:** cervical cancer; human papillomavirus; E6; E7; chemokine; monocyte chemoattractant protein-1

HPVs are etiologically involved in carcinoma of the uterine cervix. Among the nearly 90 HPV serotypes isolated to date, a subset of high-risk viruses has been found in over 90% of human cervical cancers.<sup>1</sup> These include HPV16 (detected in 50% of tumors), HPV18, HPV31 and HPV33. The products of the viral oncogenes E6 and E7 from high-risk viruses have been implicated in carcinogenesis because their expression is sufficient for the immortalization of primary human keratinocytes *in vitro*.<sup>2–4</sup> E6 targets p53 for ubiquitin-mediated degradation, while E7 binds and inactivates the product of the tumor-suppressor gene Rb.<sup>5,6</sup>

Despite this efficient program for inactivating tumor-suppressor proteins, development of cervical carcinoma is a multistep process for which E6 and E7 are necessary but not sufficient. Other genetic changes are required, which may be a consequence, in part, of the chromosomal instability produced by expression of these oncogenes.<sup>7</sup> In addition, however, the host can mount an immune response against HPV, which keeps the infection in a clinically latent state, and this latency must be overcome for cervical cancer to appear.<sup>1,8–10</sup>

Very little is known about the regulation of the immune response directed against HPV-infected epithelial cells. Although epithelial cells can present antigen and release proinflammatory cytokines when activated,<sup>11–14</sup> there is little or no inflammation at the site of primary HPV infection,<sup>8</sup> and the few inflammatory cells that are present appear not to be activated.<sup>15</sup> In addition, as epithelial dysplasia worsens during progression toward cervical

carcinoma, the cervical mucosa is gradually depleted of macrophages, T cells and Langerhans cells.<sup>16</sup> These observations suggest that HPV is capable of suppressing the host's immune and inflammatory responses to viral infection and transformation.

One of the earliest cellular responses to injury or infection is the release of chemokines, which are low m.w. chemoattractant proteins that elicit local infiltration of inflammatory and immune cells.<sup>17</sup> The chemokine MCP-1 is particularly relevant in the setting of viral infection because of its ability to attract monocytes, memory T cells and NK cells *in vivo*.<sup>18</sup> However, even though MCP-1 expression is induced after infection by a variety of RNA and DNA viruses, MCP-1 expression is suppressed after epithelial cell infection by HPV *in vitro*.<sup>19</sup> This parallels the situation *in vivo*, where advancing cervical intraepithelial neoplasia is associated with loss of MCP-1 expression.<sup>20</sup>

The mechanisms responsible for HPV-mediated suppression of MCP-1 expression are unclear. The present study was undertaken to examine the effects of HPV E6 and E7 on chemokine expression by primary epithelial cells of the reproductive tract. Surprisingly, we found that viral oncogenes selectively render MCP-1 unresponsive to induction by proinflammatory cytokines and that this refractoriness is also seen in most cervical carcinoma cell lines. Our results suggest that suppression of MCP-1 may be a common and important mechanism that shields developing cervical carcinomas from the immune system.

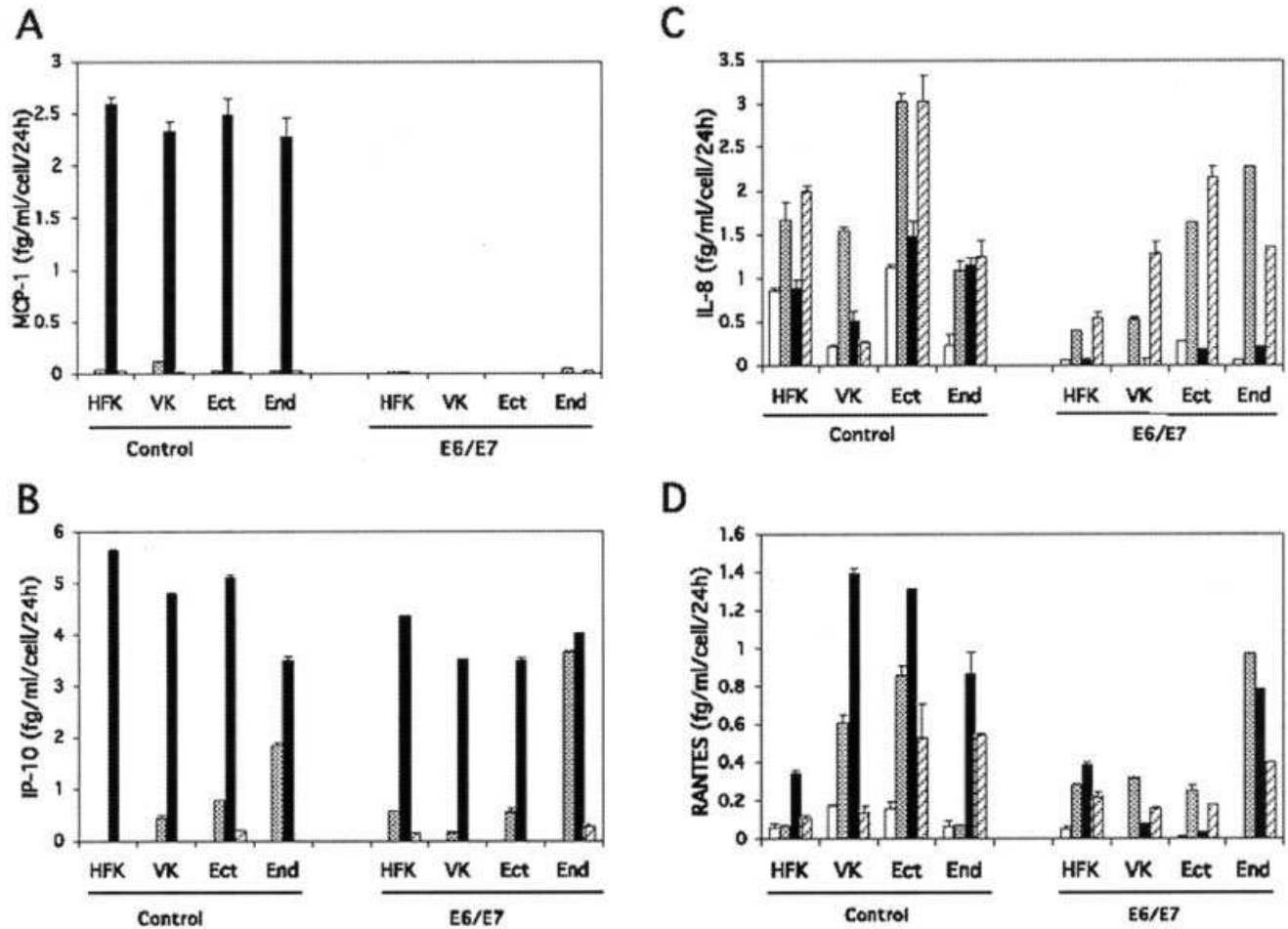
**Abbreviations:** Ab, antibody; BCS, bovine calf serum; EGF, epidermal growth factor; HFK, human foreskin keratinocyte; HPV, human papillomavirus; KGM, keratinocyte growth medium; K-SFM, keratinocyte serum-free medium; MCP-1, monocyte chemoattractant protein-1; NK, natural killer; PBMC, peripheral blood mononuclear cell; PD, population doublings; RANTES, regulated on activation, normal T cell expressed and secreted; Rb, retinoblastoma; TNF, tumor necrosis factor.

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**FIGURE 1**—Chemokine secretion by human keratinocytes immortalized by HPV16 E6/E7. HFKs; vaginal (Vk), ectocervical (Ect) or endocervical (End) epithelial cells; and immortalized derivatives (from other donors) expressing HPV16 E6/E7 (HFK/E6/E7, Vk/E6/E7, Ect/E6/E7 and End/E6/E7) were untreated or treated with TNF- $\alpha$  (50 ng/ml), IFN- $\gamma$  (50 ng/ml) or IL-1 $\alpha$  (50 ng/ml) for 24 hr. Supernatants were harvested and chemokine expression levels determined by ELISA. Error bars indicate the SD of duplicate measurements. This experiment is representative of 2. (a) MCP-1. (b) IP-10. (c) IL-8. (d) RANTES. White bar, no treatment; gray bar, TNF- $\alpha$ ; black bar, IFN- $\gamma$ ; hatched bar, IL-1 $\alpha$ .

#### MATERIAL AND METHODS

##### Cell culture and cytokines

Primary human vaginal keratinocytes, ectocervical keratinocytes, endocervical epithelial cells and foreskin keratinocytes were purchased from Clonetics (San Diego, CA) and cultured in serum-free KGM (Clonetics) supplemented with 5  $\mu$ g/ml insulin, 0.1 ng/ml hEGF and 30  $\mu$ g/ml bovine pituitary extract (referred to as *complete KGM*) and penicillin/streptomycin. HPV16 E6/E7 immortalized cells were derived from a different set of primary cells<sup>21</sup> and denoted *Vk2* (vaginal), *Ect1* (ectocervical), and *End1* (endocervical). HFKs expressing E6 or E7 alone were derived from the foreskin keratinocytes of 4- to 5-year-old donors. Cells were infected with amphotropic retroviruses carrying the E6, E7 or E6/E7 open reading frames of HPV16.<sup>22</sup> These cells were maintained in K-SFM (GIBCO BRL Life Technologies, Gaithersburg, MD) supplemented with 30  $\mu$ g/ml bovine pituitary extract, 0.1 ng/ml hEGF, penicillin, streptomycin and 0.4 mM CaCl<sub>2</sub>. Cervical carcinoma cell lines, including HeLa, SiHa, C33a, C4-1, Caski and MS751 (ATCC, Manassas, VA), were maintained in DMEM (GIBCO BRL) containing 10% BCS, penicillin and streptomycin. Cervical carcinoma cell lines ME180 and HT-3 (ATCC) were maintained in McCoy's 5a medium (GIBCO BRL) containing 10% BCS and penicillin/streptomycin. PBMCs were isolated from heparinized human blood using Ficoll-Hypaque (Pharmacia, Piscataway, NJ). TNF- $\alpha$ , IFN- $\mu$  and IL-1 $\alpha$  were obtained from R&D

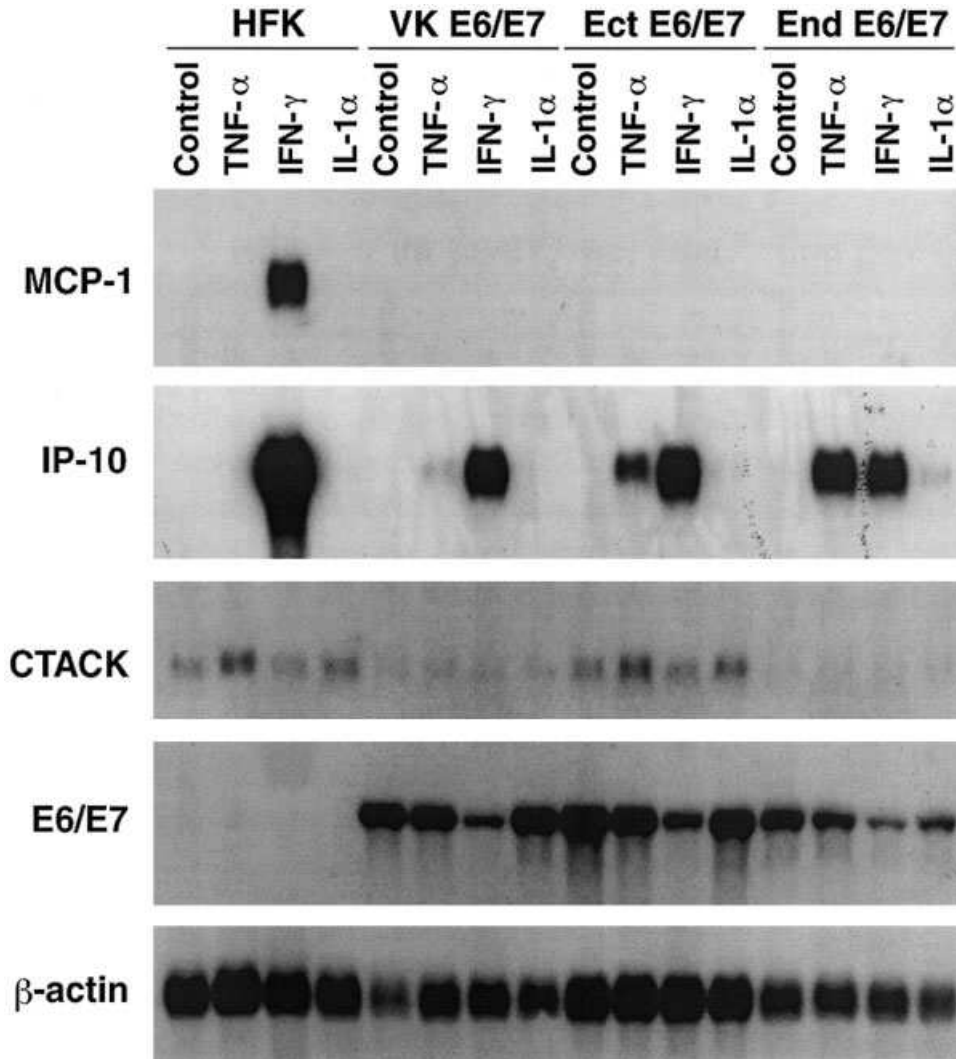
Systems (Minneapolis, MN). Cell proliferation was measured as PD, defined as log<sub>2</sub>(number of cells at subculture/number of cells plated).<sup>23</sup>

##### Preparation of retroviral stocks and retroviral infection

Amphotropic packaging cell lines (PA317) transfected with LXSN, LXSN-HPV16-E6, LXSN-HPV16-E7 and LXSN-HPV16-E6/E7 were purchased from the ATCC and grown in DMEM supplemented with 10% BCS. Conditioned media were collected, filtered and used to infect primary keratinocytes, which were selected in 100  $\mu$ g/ml G418.

##### ELISA

Cells were seeded in the appropriate medium at a density of 10<sup>6</sup> cells/100 mm dish. After 24 hr, cells were placed in low serum for an additional 24 hr, then stimulated with recombinant human cytokines for 24 hr. Conditioned media were collected, and chemokine secretion was measured using commercially available ELISA kits (R&D Systems) according to the manufacturer's instructions. Abs for the IP-10 ELISA were obtained from R&D Systems (capture, catalog MAB266; detection, catalog AF-266-NA). The concentrations of the capture and detection Abs were 4  $\mu$ g/ml and 100 ng/ml, respectively. The biotinylated detection Ab was quantified using streptavidin-conjugated horseradish peroxidase, followed by orthophenylenediamine and color development.



**FIGURE 2** – Induction of chemokine mRNA expression in primary human keratinocytes. Normal HFKs and HPV16 E6/E7-immortalized primary vaginal (Vk), ectocervical (Ect) and endocervical (End) epithelial cells were either left untreated or treated with TNF- $\alpha$  (50 ng/ml), IFN- $\gamma$  (50 ng/ml) or IL-1 $\alpha$  (50 ng/ml) for 24 hr. Cellular RNA was isolated and analyzed by Northern blotting using the indicated probes.

#### *RNase protection assay*

Total cellular RNA was isolated using RNeasy (Qiagen, Hilden, Germany). Multiple template set hCK5 was purchased from Pharmingen (San Jose, CA). DNA templates were used to synthesize probes incorporating  $\alpha$ -[ $^{32}$ P]UTP (3,000 Ci/mmol; Life Science Products, Boston, MA) using T7 polymerase. Hybridization with 15  $\mu$ g of each target RNA was performed overnight followed by digestion with RNase A and T1 according to the manufacturer's protocol. Samples were treated with a proteinase K–SDS mixture, extracted with phenol:chloroform:isoamyl alcohol (50:49:1) and then precipitated with ethanol in the presence of ammonium acetate. Samples were loaded on a 5% acrylamide-urea sequencing gel next to the labeled probes and electrophoresed at 50 W in 0.5  $\times$  TRIS-borate/EDTA buffer. The gel was dried and exposed to X-ray film.

#### *Northern blot analysis*

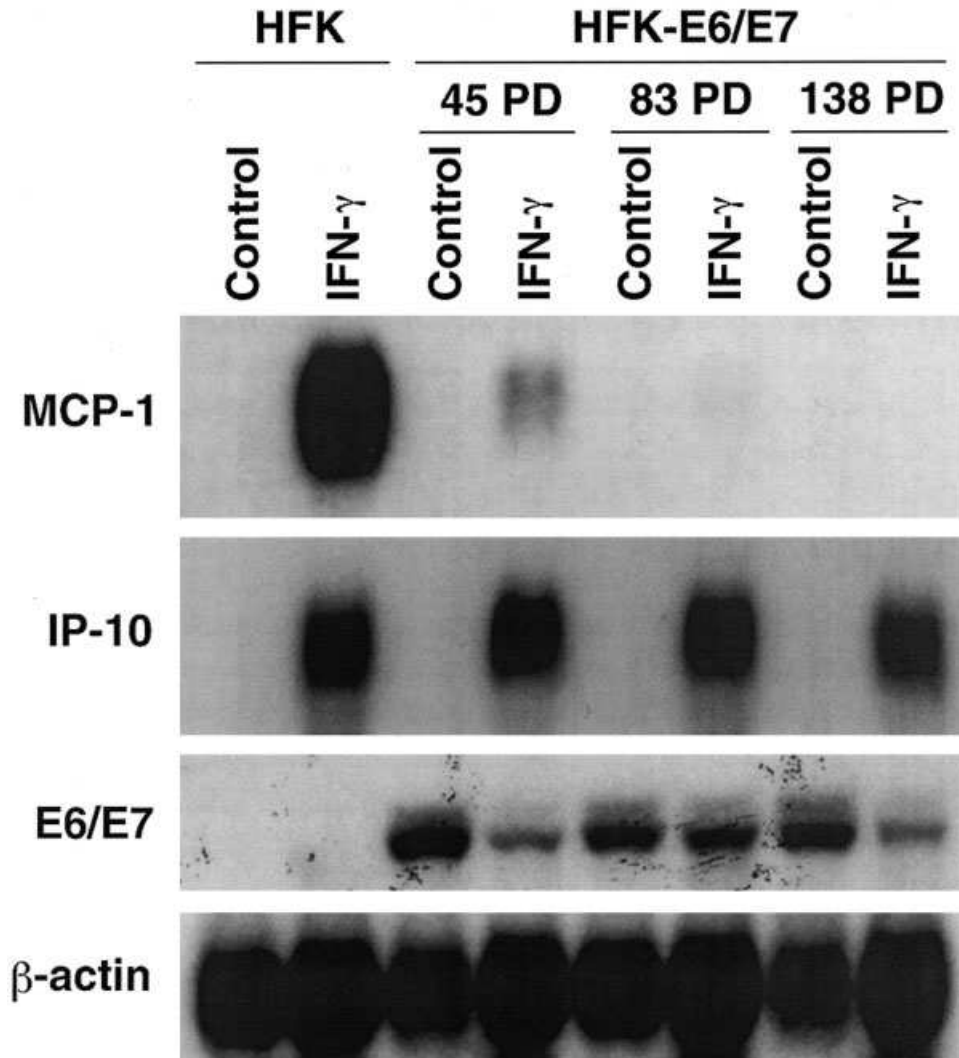
Total RNA was purified from subconfluent cells using RNeasy, and 8  $\mu$ g were fractionated by electrophoresis through a 1.2% agarose/2.2 M formaldehyde gel in 3-[*N*-morpholino]propane sulfonic acid/EDTA buffer. RNA was transferred to nylon filters (Nytran; Schleicher & Schuell, Dassel, Germany) in 10  $\times$  SSC

(1  $\times$  SSC is 0.15 M NaCl/0.015 M sodium citrate, pH7) using a TurboBlotter (Schleicher & Schuell) and crosslinked using a Stratalinker (Stratagene, La Jolla, CA). cDNA probes for MCP-1, IP-10, IL-8, RANTES, HPV16 E6/E7 and  $\beta$ -actin were radiolabeled with  $\alpha$ -[ $^{32}$ P] dCTP (3,000 Ci/mmol, Life Science Products) by random primer labeling (High Prime; Boehringer-Mannheim, Mannheim, Germany). Hybridization was carried out in ExpressHyb hybridization solution (Clontech, Palo Alto, CA) under stringent conditions. A CTACK cDNA probe was prepared by RT-PCR from primary HFKs after isolation of total RNA by RNeasy. The sequence for the sense primer was GGAA-GAGTCTAGGCTGAGCA and that of the antisense primer, GC-CATTTTCCTTAGCATCC.

#### RESULTS

##### *Chemokine expression in epithelial cells in the presence and absence of HPV E6/E7*

HFKs, vaginal and ectocervical keratinocytes and endocervical epithelial cells were tested for expression of 4 chemokines involved in innate and/or adaptive immune responses (Fig. 1). Under the culture conditions used here, all cells constitutively secreted



**FIGURE 3** – Chemokine mRNA expression with continued proliferation after HPV16 E6/E7 expression. HFKs were transduced to express HPV16 E6/E7 at 33 PD. After the indicated number of PD, RNA was harvested and analyzed by Northern blotting.

low to undetectable amounts of MCP-1, IP-10 and RANTES and moderate amounts of IL-8. However, cytokine treatment induced high levels of expression of all 4 chemokines. In particular, IFN- $\gamma$  induced abundant MCP-1, IP-10 and RANTES secretion, while TNF- $\alpha$  and IL-1 $\alpha$  were better inducers of IL-8.

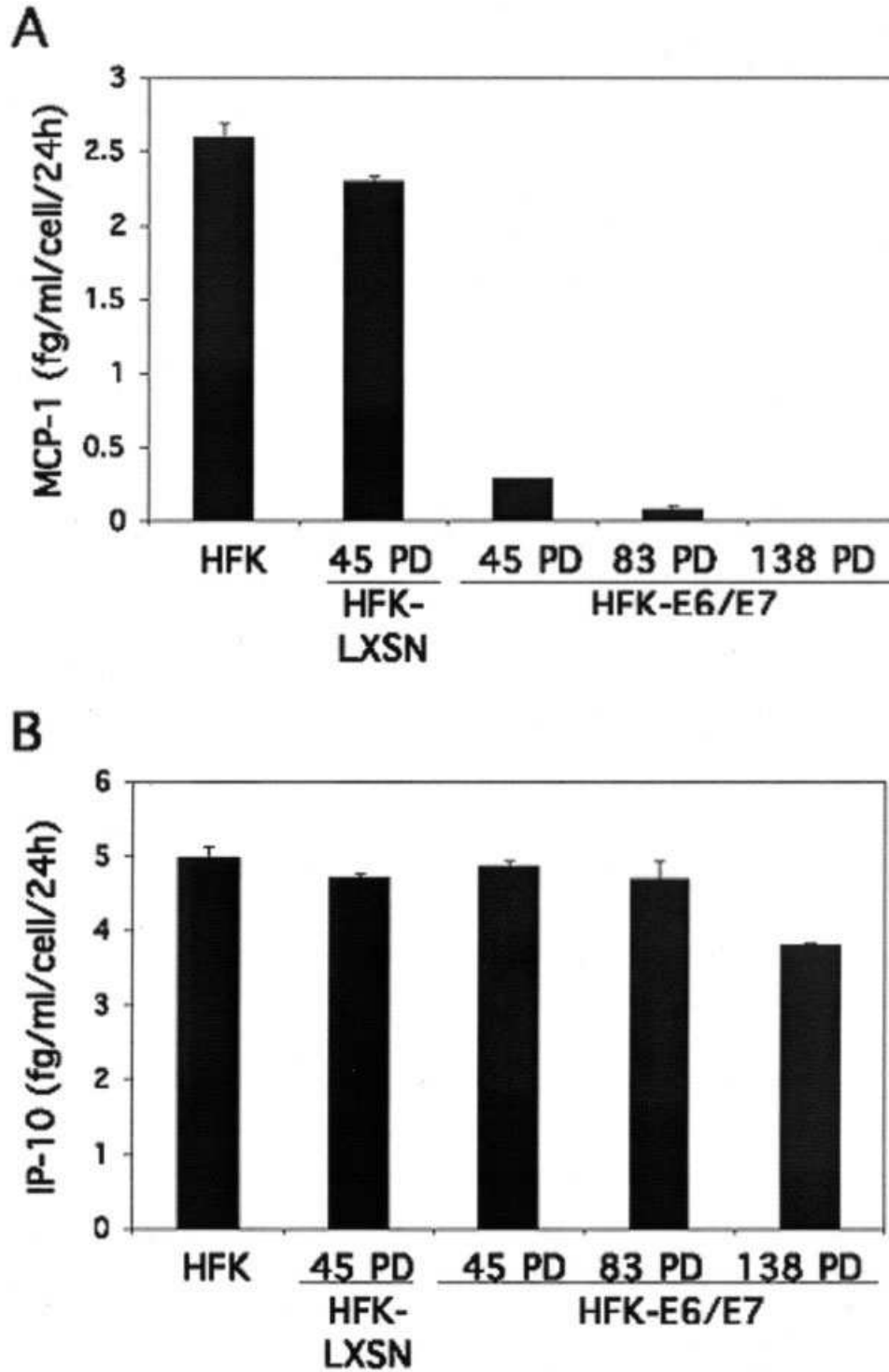
To determine whether high-risk HPV E6/E7 suppresses chemokine expression, we examined cells of similar origin that were immortalized by retroviral transduction of HPV16 E6/E7<sup>21</sup> and maintained in culture for >120 PD. As shown in Figure 1a, none of these cells secreted detectable levels of MCP-1 in response to IFN- $\gamma$ , which was a potent inducer of MCP-1 in cells that do not express E6/E7. Nonetheless, all of the E6/E7-expressing cells secreted IP-10 in response to IFN- $\gamma$  (Fig. 1b), indicating that they had functional IFN- $\gamma$  receptors and that at least some signal-transduction pathways from this receptor were intact. Similarly, secretion of IL-8 and RANTES in response to IFN- $\gamma$ , TNF- $\alpha$  or IL-1 $\alpha$  was suppressed to a much lesser extent or not at all by E6/E7 expression (Fig. 1c,d). This provides further evidence for the specific suppression of MCP-1 by E6/E7.

Like the ELISA results in Figure 1, the Northern blots in Figure 2 show that E6/E7-transduced cells cannot express MCP-1 mRNA in response to cytokine treatment. Again, however, IP-10 mRNA can be

induced by IFN- $\gamma$  and, to a lesser extent, by TNF- $\alpha$ , as can the mRNA encoding CTACK, a keratinocyte-derived chemokine involved in the trafficking of T lymphocytes to mucocutaneous sites.<sup>24</sup> Thus, the specific suppression of MCP-1 by E6/E7 is exerted at the level of mRNA expression. All experiments using these primary cells were performed at least twice, with similar results.

#### *Complete suppression of MCP-1 requires prolonged culture after E6/E7 transduction*

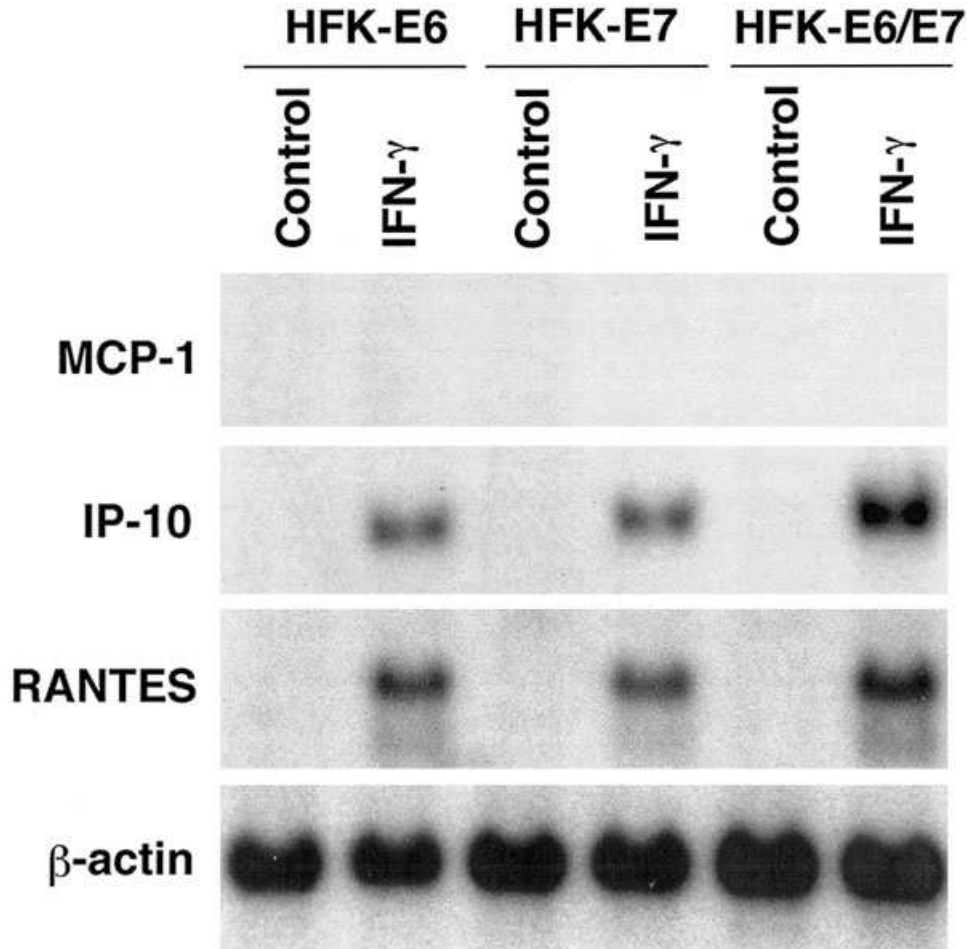
To determine whether the effects of E6/E7 on MCP-1 expression were immediate, HFKs were examined at various times after E6/E7 transduction. HFKs were infected with LXS-E6/E7 at approximately 33 PD. Figure 3 shows that at 45 PD (approx. 3 weeks after transduction and G418 selection), a small amount of MCP-1 mRNA expression could be induced in response to IFN- $\gamma$ . At 83 PD, a trace signal of IFN- $\gamma$ -inducible MCP-1 mRNA expression was still present. Not until 138 PD was MCP-1 expression undetectable after IFN- $\gamma$  treatment. This gradual loss of MCP-1 inducibility was not an artifact related to persistence of cells uninfected by retrovirus since selection for G418 resistance was complete within 5 PD after transduction. Furthermore, the level of E6/E7 transcription in untreated cells did not increase from 45 PD



**FIGURE 4** – Chemokine secretion by human keratinocytes with continued passage after HPV16 E6/E7 expression. HFKs were infected with LXSN- or LXSN-expressing HPV16 E6/E7. After the indicated PD, cells were either left untreated or treated with IFN- $\gamma$  (50 ng/ml) for 24 hr. Supernatants were harvested and concentrations of MCP-1 (a) and IP-10 (b) measured by ELISA. Error bars indicate the SD of duplicate measurements. This experiment is representative of 2.

onward. Again, specificity for MCP-1 was demonstrated by similar levels of IP-10 mRNA expression induced by IFN- $\gamma$  at all passages. Analysis of chemokine secretion from these cells showed that levels of MCP-1 and IP-10 protein in conditioned medium

corresponded to levels of mRNA expression (Fig. 4). Notably, suppression of inducible MCP-1 expression could be achieved by expression of either E6 or E7 alone as well as the combination of both viral oncogenes (Fig. 5).



**FIGURE 5** – MCP-1 mRNA expression is suppressed in HFKs by E6 or E7 alone as well as E6/E7 in combination. HFKs were infected with LXS<sup>N</sup>- or LXS<sup>N</sup>-expressing HPV E6, E7, or E6/E7 as described in Material and Methods and tested for IFN- $\gamma$ -induced chemokine mRNA expression by Northern blotting.

#### *Chemokine expression in cervical carcinoma cell lines*

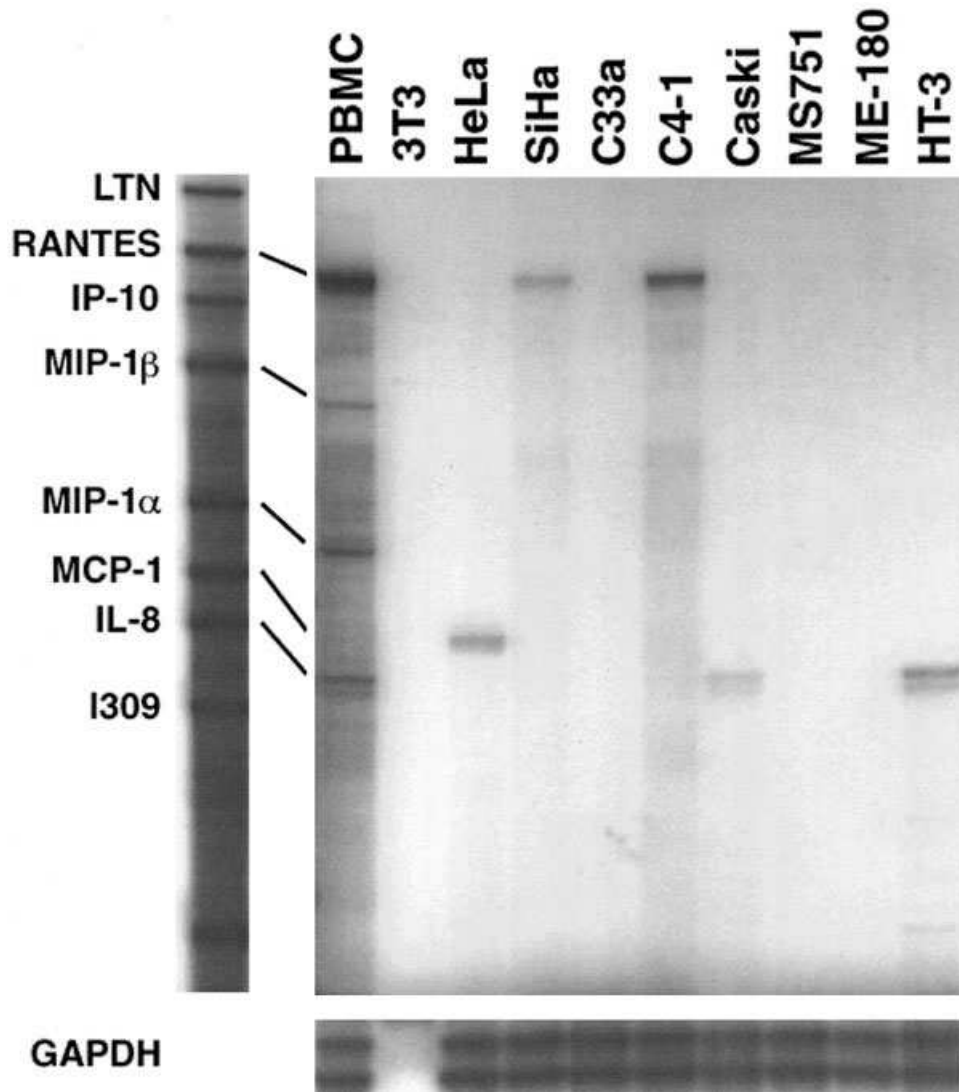
To determine whether patterns of E6 and/or E7 suppression of chemokine expression in primary cells transduced in culture also occurred in HPV-associated tumors, several cervical carcinoma cell lines were examined. Figure 6 shows that some cell lines constitutively transcribed chemokine mRNA. In particular, HeLa cells expressed MCP-1, SiHa and C4-1 cells expressed RANTES and Caski and HT-3 cells expressed IL-8. (HFKs expressed no chemokine mRNAs constitutively, but in response to IFN- $\gamma$  they transcribed IP-10 and MCP-1 predominantly and RANTES to a lesser extent.)

Figure 7 shows a more thorough examination of MCP-1, IP-10, IL-8 and RANTES secretion in response to cytokines. Consistent with the results in Figure 6, only HeLa cells expressed MCP-1 protein constitutively, and secreted amounts were not increased by treatment with TNF- $\alpha$ , IFN- $\gamma$  or IL-1 $\alpha$ . Of the other 7 cervical carcinoma cell lines tested, only SiHa showed inducible MCP-1 expression in response to IFN- $\gamma$ . Thus, only 2 of 8 lines expressed constitutive or inducible MCP-1. In contrast, 5 of 8 lines secreted abundant IP-10 in response to IFN- $\gamma$ . Most of the cell lines, except for C33a, also expressed IL-8 or RANTES in response to cytokine induction. All but 2 of these cell lines, C33a and HT-3, are HPV-positive, so these results generally corresponded to chemokine expression patterns observed in E6- and/or E7-expressing primary cells.

#### DISCUSSION

We examined patterns of chemokine expression by epithelial cells of the female reproductive tract and found that primary vaginal, endocervical and ectocervical cells express MCP-1, IP-10, IL-8 and RANTES in response to inflammatory cytokines. After transduction of both E6 and E7 from high-risk HPV, however, these cells no longer expressed MCP-1 in response to any inducer but were still able to express other chemokines. Selective loss of MCP-1 expression was also observed in 4 of 6 HPV-positive cervical carcinoma cell lines, though this must be interpreted with caution because of potential differences between these cell lines and authentic cervical carcinomas. MCP-1 expression by HeLa cells in our study contradicts earlier reports.<sup>19</sup> Although the lineage history of the HeLa culture we examined may differ from that used in the earlier study, the HPV genome is still present in the cells we tested (data not shown) and their ability to secrete MCP-1 is unexplained. Nonetheless, in primary epithelial cells, the correlation between E6/E7 expression and suppression of MCP-1 is complete.

Interpretation of these data is somewhat limited by the fact that the E6/E7-transduced and nontransduced cells did not come from the same individuals; therefore, differences in genetic background may have accounted for disparities in MCP-1 expression. However, the 4 nontransduced cells came from 4 independent donors as did the 4 transduced cells, and the patterns of MCP-1 expression and suppression are consistent across all lines. This suggests that



**FIGURE 6**—Chemokine mRNA expression by cervical carcinoma cell lines. Cells were grown under standard conditions as described in Material and Methods. RNA was isolated and analyzed for chemokine expression by RNase protection assay.

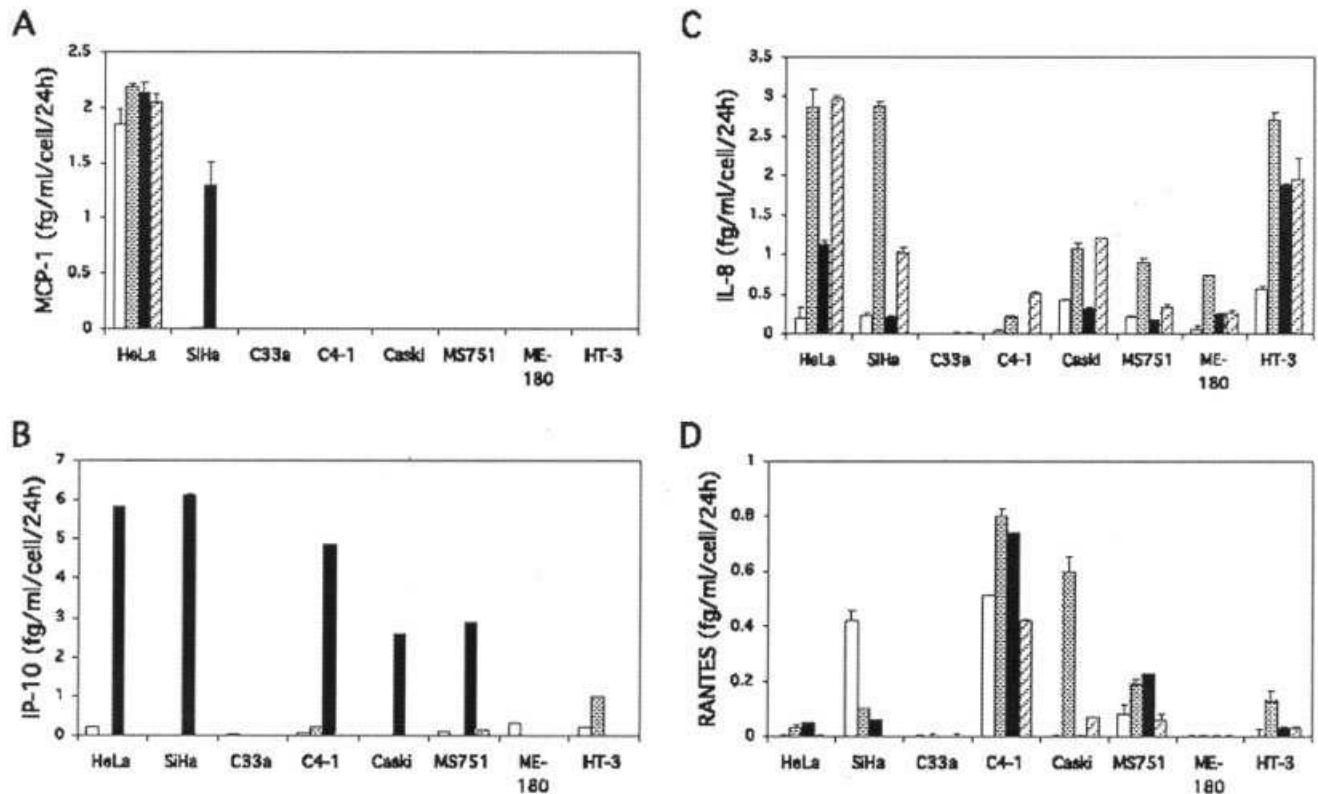
suppression of MCP-1 expression by E6 and E7 is a generalizable phenomenon that is independent of specific genetic background.

The mechanisms underlying suppression of inducible MCP-1 transcription are only partly understood. Finzer et al.<sup>25</sup> documented a DNase hypersensitive site in a 3' domain that contains an AP-1 binding motif and showed that the composition of the *trans*-acting heterodimer that binds to this site differs between cells that do and do not express MCP-1. Another possible mechanism is based on analysis of differences between upstream promoter regions of MCP-1 and IP-10. In the *MCP-1* gene, a 5' domain AP-1 binding site overlaps an SP-1 binding GC box,<sup>26–28</sup> but the GC box is absent from the IP-10 promoter.<sup>28</sup> Since this site is important for IFN- $\gamma$ -inducible MCP-1 expression, disruption of this transcriptional control element by E6 and E7 might differentially affect MCP-1 and IP-10 expression, as observed in the present report. Any such outcome must be the result of concerted effects by E6 and E7 together since either viral oncogene alone could not suppress MCP-1 expression. Determining whether this is a direct result of Rb and p53 inactivation as opposed to other activities of E6 and E7 will be the focus of future studies.

Regardless of mechanism, our data indicate that expression of E6 and E7 did not immediately result in MCP-1 suppression (Figs.

3, 4). While it is possible that this is a consequence of the persistence of E6/E7-negative cells in culture, cells had been transduced 3 weeks prior to the analysis at 45 PD. The selective pressure of G418 in the medium and the proliferative advantage imparted by E6/E7 expression make this unlikely and suggest that these viral oncogenes are necessary but not sufficient for MCP-1 suppression. On the one hand, this may simply be a reflection of heterogeneity in the effectiveness of oncogene-mediated inhibition of expression, which becomes uniform with continued serial passage. On the other hand, it may reflect fundamental changes that occur during progression toward immortalization or during the development of the fully transformed phenotype. In this regard, one possible mechanism for the eventual suppression of MCP-1 is that the chromatin of the MCP-1 locus gradually undergoes remodeling by DNA methylation or histone deacetylation when E6 and E7 are expressed. However, treatment of E6/E7-expressing cells with 5-aza-2'-deoxycytidine or trichostatin A did not restore MCP-1 expression (data not shown). Thus, it is more likely that additional genetic, rather than epigenetic, changes occur in cells containing high-risk HPV oncogenes and that one of the consequences of these changes is loss of MCP-1 expression. Furthermore, there are almost certainly other pathways to MCP-1 sup-





**FIGURE 7** – Chemokine secretion by cervical carcinoma cell lines. Cells were untreated or treated with TNF- $\alpha$  (50 ng/ml), IFN- $\gamma$  (50 ng/ml) or IL-1 $\alpha$  (50 ng/ml) for 24 hr. Supernatants were harvested and chemokine expression levels determined by ELISA. Error bars indicate the SD of duplicate measurements. This experiment is representative of 2. (a) MCP-1. (b) IP-10. (c) IL-8. (d) RANTES. White bar, no treatment; gray bar, TNF- $\alpha$ ; black bar, IFN- $\gamma$ ; hatched bar, IL-1 $\alpha$ .

pression that do not involve *E6/E7* since some of the cervical carcinoma cell lines that do not express MCP-1 also do not express HPV oncogenes, e.g., C33a and HT-3.<sup>29</sup>

The selective loss of MCP-1 expression after *E6/E7* transduction may have important implications for the pathogenesis of cervical carcinoma. MCP-1 is a potent chemoattractant for monocytes, macrophages and NK cells, all of which could be involved in the host's immunologic response to HPV-infected cervical epithelium.<sup>18</sup> Gene transfer experiments have also shown that MCP-1 can produce an immunologically specific cytotoxic response against syngeneic tumors *in vivo*.<sup>30</sup> Suppression of MCP-1 secretion would blunt these responses and give infected cells sufficient time to accumulate additional genetic changes necessary for the development of invasive carcinoma. Our findings may explain the absence of a vigorous inflammatory response to primary HPV infection in humans as well as the gradual loss of immune cells from the cervical stroma during the progression of cervical intraepithelial neoplasia<sup>8,16</sup> since most of these infiltrating cells express the primary MCP-1 receptor, CCR2.

Our results support the general notion that immunotherapy may be particularly effective in carcinoma of the cervix. As noted by

others, the presence of viral products in these cells would lead to the expression of tumor antigens,<sup>31</sup> and a cell-mediated immune response to high-risk *E6/E7* protein products has been suggested to protect against advancing disease.<sup>32</sup> It is likely that suppression of MCP-1 was evolutionarily selected precisely to render cells expressing these neoantigens less susceptible to host recognition. The targeting of MCP-1 by high-risk HPV is an indication that this chemokine may be particularly important in the host response to infected cells. Restoring MCP-1 expression locally, by genetic or direct protein application methods or by including it in a therapeutic tumor vaccine, may specifically enhance host antitumor effector mechanisms.

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