TOPIC HIGHLIGHT

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Role of IL-10 and TGF-β1 in local immunosuppression in HPV-associated cervical neoplasia

Kirvis Torres-Poveda, Margarita Bahena-Román, Claudia Madrid-González, Ana I Burguete-García, Víctor Hugo Bermúdez-Morales, Oscar Peralta-Zaragoza, Vicente Madrid-Marina

Kirvis Torres-Poveda, Margarita Bahena-Román, Claudia Madrid-González, Ana I Burguete-García, Víctor Hugo Bermúdez-Morales, Oscar Peralta-Zaragoza, Vicente Madrid-Marina, Chronic Infection and Cancer Division, Research Center on Infectious Diseases, Instituto Nacional de Salud Pública, 62100 Cuernavaca, Morelos, Mexico

Author contributions: Torres-Poveda K and Madrid-Marina V participated in the design of the review and drafted the manuscript; Bahena-Román M, Madrid-González C, Burguete-García AI, Bermúdez-Morales VH and Peralta-Zaragoza O participated in the redaction of results previous for this review; Bermúdez-Morales VH participated in the design of figure 1; all authors read and approved the final manuscript.

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Correspondence to: Vicente Madrid-Marina, MD, PhD, Chronic Infection and Cancer Division, Research Center on Infectious Diseases, Instituto Nacional de Salud Pública, Avenida Universidad 655, Col. Santa María Ahuacatitlán, 62100 Cuernavaca, Morelos. Mexico. vmarina@insp.mx
Telephone: +52-777-3293056 Fax: +52-777-3293056
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Abstract

Cervical cancer is a worldwide disease that constitutes a significant public health problem, especially in developing countries, not only due to its high incidence but also because the most affected population comprises women who belong to marginalized socio-economic classes. Clinical and molecular research has identified immunological impairment in squamous intraepithelial cervical lesions and cervical cancer patients. Human Papillomavirus (HPV) has several mechanisms for avoiding the immune system: it down-regulates the expression of interferon and upregulates interleukin (IL)-10 and transforming growth factor (TGF)-β1 to produce a local immunosuppressive environment, which, along with altered tumor surface antigens, forms an immunosuppressive network that inhibits the antitumor immune response. In this review we analyzed the available data on several deregulated cellular immune functions in patients with NIC Ⅰ, NIC Ⅱ and NIC Ⅲ and cervical cancer. The effects of immunosuppressive cytokines on innate immune response, T-cell activation and cellular factors that promote tumor cell proliferation in cervical cancer patients are summarized. We discuss the functional consequences of HPV E2, E6, and E7 protein interactions with IL-10 and TGF-β1 promoters in the induction of these cytokines and postulate its effect on the cellular immune response in squamous intraepithelial cervical lesions and cervical cancer patients. This review provides a comprehensive picture of the immunological functions of IL-10 and TGF-β1 in response to HPV in humans.

Core tip: Human Papillomavirus (HPV) persistence is a key event for cervical cancer development. HPV proteins E2, E6 and E7 induce the transcription of immunosuppressive cytokines as a means of evading HPV the host immune system. We postulate that interleukin-10 and transforming growth factor-β1 induce HPV immune system evasion through an immunosuppressive state in the local microenvironment of the cervix in HPV-infected women. These findings allow us to gain insight in our understanding of how HPV persist in the cervix and favor cervical lesions and cancer development, with potentially strong impact on vaccine development and the

design of new targeted immunotherapies for women with cervical neoplasia.

INTRODUCTION

Cervical cancer is the second most common cancer in women worldwide[1]. Cervical cancer and its precursor, squamous intraepithelial lesion (SIL), arise as a result of an uncontrolled and persistent infection with a high-risk human Papillomavirus (HPV)[2]. Based on molecular epidemiological studies that provide risk estimates of specific HPV types in cases and controls, as well as the evidence of the oncogenic potential of the different HPV types, 13 high-risk HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, and 58, 68, 82) have been identified, with three considered probable (26, 53, 66). Ten types were classified as low-risk (6, 11, 40, 42, 43, 44, 54, 61, 72, 81CP6 108) and three of indeterminate risk (34, 57, and 83)[3]. The most prevalent high-risk viral types in the general population are 16 and 18. These two types are responsible for approximately 70% of all cervical cancer cases[4].

Most of the HPV infections are of a transient and intermittent nature, especially among women under 30 years of age[5]. Around 70% of infections disappear within approximately one year. After two years 90% of the infections will have disappeared. Only 10% of the women studied remained infected and developed cervical epithelium alterations and eventually cervical cancer[5,6]. The majority of women clear HPV infections spontaneously through the antiviral host immune response. The mechanistic explanation for HPV clearance is by specific immunological reactions, which require competent humoral and cell-mediated immune mediators[7,8]. Among the factors influencing viral persistence are host factors (genetic or acquired, such as age, immunosuppression, oral contraception, smoking) and viral factors (genotype, variants, viral load and viral integration)[6,9].

In this review we discuss the mechanisms that allow HPV and human cervical cancer cells to evade immune surveillance through soluble immunosuppressive factors produced in the tumor microenvironment, such as interleukin (IL)-10 and transforming growth factor (TGF)-β1, and we dissect the molecular events underlying tumor immune escape.

NATURAL HISTORY OF CERVICAL CANCER

The natural history of cervical cancer has been well established by a large number of prospective cohort studies. Figure 1 summarizes the molecular events and changes at tissue level related to HPV infection and cancer establishment.

The development of a precancerous lesion and cancer involves several events. Exposure to high-risk HPV causes an initial infection of squamous epithelium in the transformation zone. This is followed by persistent infection, viral genome integration into the host cell genome, genomic alterations, immortalization and transformation of epithelial cells (Figure 1)[10].

As the understanding of the natural history of the disease has improved, the classification of these lesions has received different names [PAP Ⅰ to Ⅴ; moderate dysplasia, carcinoma *in situ* and severe, cervical intraepithelial neoplasia (CIN) Ⅰ, Ⅱ, Ⅲ; low squamous intraepithelial lesions (LSIL) that include CIN I or mild dysplasia, condyloma and koilocytosis and high squamous intraepithelial lesions (HSIL) that include CIN Ⅱ and CIN Ⅲ or moderate or severe dysplasia and carcinoma *in situ*], as shown in Figure 1. The development of cervical cancer is preceded by a series of cellular abnormalities characterized by cytological and histological maturation variations and irregularities in nuclear cytoplasm[5]. LSIL have a diploid DNA content or polyploid. This is correlated with their tendency to revert. In contrast, HSIL type CIN Ⅲ is often aneuploid, have a greater degree of cellular atypia and are more likely to persist and progress[6].

The immune system plays a key role during HPV-carcinogenesis since the majority of high-risk HPV infections (90%), as well as most of low-grade lesions (75%), regress. Upon infection, on average 2-3 years are necessary to develop CIN 1/2 and/or high-grade intraepithelial lesion (CIN3) and nearly one third of untreated HSIL may progress to cancer in about ten years[5,6].

HPV enters the mitotically active basal cells through micro abrasions in the epithelium of the cervix. After infection, early HPV genes E1, E2, E4, E5, E6 and E7 are expressed in infected cells and the viral genome is maintained episomally. The virus can lie latent until the negative regulation of viral transcription in these cells by cellular factors is released. The viral genome is replicated with greater intensity and late genes L1 and L2 are expressed in the upper layers of the epithelium. Capsid ruptures allow the release of the viral genome, the formation of new virions and the start of a new infection[11,12].

The long latency period between initial infections and the emergence of cancer suggests that HPV can evade recognition by the immune system. Indeed, the infection cycle of HPV is characterized by the absence of viraemia, very low expression levels of viral protein, no inflammation and no danger signal to alert the immune system[13-15]. Host immune responses to HPV are generally low-level because the virus, being confined to basal epithelial cells, is shielded from the circulating immune cells during initial stages of infection. In this location there is only a limited expression of viral proteins. Other factors contributing to the low level of host immunity are that HPV infection
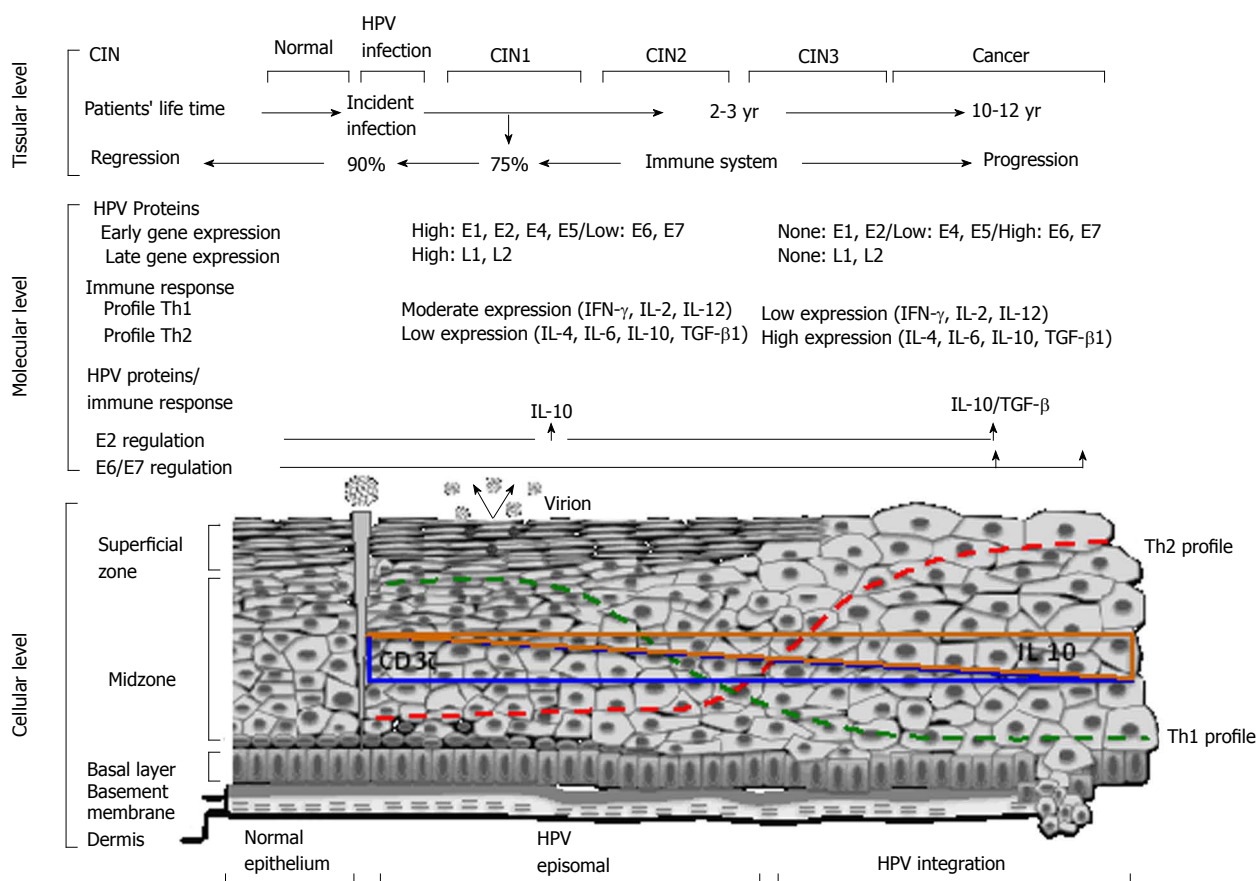
Figure 1 Systematic representation for cervical human Papillomavirus-carcinogenesis: Role of immune response. The main event is the human Papillomavirus (HPV) infection of epithelial cells in the basal membrane. HPV can turn into episomal bodies, which will be in charge of, on one part, producing infective virions and, on the other, integrating into the genome of epithelial cells. Upon infection, an average of 2-3 yr are necessary to develop of CIN 1/2 and/or high-grade (CIN3), often characterized by the integration of the viral genome, one key event for disease progression as this frequently triggers the deregulation of the E6 and E7 oncogenes, major chromosomal alterations and cellular immortalization[10]. The immune system plays a key role during HPV-carcinogenesis since the majority of high-risk HPV infections (90%), as well as most of low-grade lesions (75%) regress[5,6]. Due to this, and the long periods of time between viral infection and the progression to invasive disease, the fail of immune response is necessary to cancer development. Since the beginning of HPV infection, there are augment in the immunosuppressive cytokine IL-10 at the cervical level, and this increased according with the grade of lesion, being the highest concentration at cancer stage[25]. A shift to Th2-type cytokines in the course of development of cervical cancer is reflected with an increased serum concentration of Th2-type cytokines[22,23]. Inversely, the presence of CD3zeta chain in the tumor infiltrating T-lymphocytes, decreases as the grade of lesion progress, this is due to a reduction of Th1-type cytokines at the late stages of the disease[24]. These are key elements for the impairment of immune responses that allows HPV-persistence, viral integration into the genome epithelial cells, and cellular transformation and immortalization.

is non-lytic; that a functionally active immune response is generated only at later stages of HPV infection; and that only in suprabasal keratinocytes has the HPV DNA been sufficiently amplified to be detected by the host immune-surveillance cells[16].

Although most HPV infections are transient and subclinical, progression is strongly associated with HPV persistence. This process often leads to disruption of viral E1/E2 regions and integration in the genome of the host cell. E2 rupture releases E6/E7 viral promoters and increases expression of these oncogenes, as show in Figure 1[10-12,17]. Infection with one high-risk HPV acts as a trigger for the cascade of events in which the mechanisms of repair or correction of cell replication, mediated by p53 and pRb, are altered. Thus, the cell cycle is controlled by the virus, which triggers cellular changes that culminate in the transformation and immortalization of epithelial cells, in consequence establishing conditions for the onset of cancer[18-20].

During cancer progression, the pattern of changes in expression of viral oncoproteins changes; in CIN I, the order of events is similar to that observed in productive lesions. In CIN II and III, however, the event occurrence is delayed and virions production is restricted to smaller areas near the epithelial surface. Integration of HPV sequences in the genome of the host cell may accompany these changes and may lead to a further deregulation of the expression of E7 (and the loss of replication proteins E1 and E2). In cervical cancer, the production stages of the viral cycle are not permanent and the viral episomes are lost[11,12].

A predominance of the Th2 cytokine profile, in association with a diminished Th1 profile, has been demonstrated in patients with cervical cancer[21]. A shift to Th2-type cytokines in the course of development of cervical cancer is reflected by an increased serum concentration

of Th2-type cytokines[22,23]. Inversely, the presence of CD3zeta chains in the tumor-infiltrating T-lymphocytes decreases as the grade of lesion progresses. This is due to a reduction of Th1-type cytokines in the late stages of the disease[24]. This shift from Th1 to Th2 might be responsible for facilitating tumor progression by subverting various cellular immune surveillance mechanisms.

In addition, an impaired cellular immune response induced by immune suppressor cytokines, such as interleukin (IL)-10 and transforming growth factor beta (TGF-β1), has been involved in high risk HPV persistence and cervical cancer development[21]. Since the beginning of HPV infection there are increases in the immunosuppressive cytokine IL-10 at the cervical level. The increase is proportional to the grade of lesion, with the highest concentration occurring at the cancer stage[25]. These are key elements for the impairment of immune responses that enable HPV-persistence, viral integration into the genome epithelial cells, and cellular transformation and immortalization.

IMMUNE EVASION IN HPV INFECTION, SIL AND CERVICAL CANCER

Innate immune response, which involves macrophages, natural killer (NK) cells, and natural killer T cells, plays a critical role as the first line of defense against HPV infection[26]. However, effective evasion of innate immune recognition seems to be the hallmark of HPV infections[27]. Several alterations of natural immunity have been documented in HPV infection. The interferon (IFN) response, a key antiviral defense mechanism[28], is actively suppressed by the E6 and E7 proteins of high-risk HPVs, which inhibit the interferon receptor signaling pathways and prevent activation of the interferon response genes[29-31]. Macrophages are activated by binding to viral components, such as single-stranded DNA, and by cytokines, which can kill HPV-infected cells via tumor necrosis factor α (TNF-α) secretion. HPV16 E6 and E7 proteins inhibit the translocation of macrophages to the site of HPV infection[32].

Likewise, NK cells are a subset of lymphocytes that kill virally infected cells or tumor cells lacking the surface expression of major histocompatibility complex (MHC) class-1 molecules. Low NK cell receptor expression and reduced cytotoxic activity of NK cells were recently observed in cervical cancer and precursor lesions. A previous study suggests that NKp30, NKp46 and NKG2D down-regulation represents an evasion mechanism associated with low NK cell activity, HPV-16 infection and cervical cancer progression[26,33]. Recent studies have demonstrated that CD1d on the surfaces of cells infected with HPV6 or 16 is down-regulated by E5 protein, and that this may be a mechanism for immune evasion[34]. Additionally, augmented serum level of major histocompatibility complex class I-related chain A (MICA) protein and reduced NKG2D expression on NK and T cells in patients with cervical cancer and precursor lesions have

been reported[35,36].

Similarly, Toll-like receptors (TLRs) play a key role in the innate immune system against HPV infection. The activation of the TLR9 pathway by CpG motives is impaired severely in human keratinocytes expressing HPV16 E6 and E7 oncoproteins. This event is due to the ability of the viral oncoproteins to down-regulate TLR9 mRNA. This phenomenon has been observed in HPV16-positive cancer-derived cell lines and in primary cervical cancers. TLR9 promoter down-regulation is less significant for high-risk HPV 18 compared with HPV 16 and is completely absent in cells expressing E6 and E7 from the low-risk HPV 6. Thus, the efficiency of HPV16 in persisting appears to correlate with its ability to down-regulate the transcription of TLR9[37].

In high-risk HPV-infected women there is a decrease in the levels of Langerhans DCs in the human female genital tract (reduction of E-cadherin by E6)[38]. CD4 regulatory T cells (Treg) subsets act as major mediators of peripheral immune tolerance by regulating Th1 and Th2 immune responses[39,40]. Treg cells contribute to the induction of peripheral tolerance via expression of inhibitory cell-surface molecules ($CD4^+/CD25^+$ T cells) or production of IL-10 and TGF-β1. IL-10 produced by Treg cells can impair TAA cross-presentation by DCs, thus potentially preventing T cells from mounting an effective immune response against malignant cells. IL-10 hinders the antigen-presenting properties of DCs by reducing their expression of *human leukocyte antigen* (*HLA*) class II molecules, inter-cellular adhesion molecules (*e.g.*, ICAM-1), co-stimulatory molecules (*i.e.*, B7-1/CD80 and B7-2/CD86), and Th1 cytokines (*e.g.*, IL-12), which correlates with the ability of IL-10 to impair primary alloantigen-specific T cell responses[41]. IL-10 inhibits the host inflammatory response and favors tumor development. Thus, the predominant secretion of Th2 cytokines in innate immunity attenuates the antitumoral response[42].

In addition, HPV effectively evades the innate immune response to delay the activation of adaptive immunity in patients with SIL[43-45]. HPV16 E5-mediated immune evasion also involves suppressing the expression of MHC class I and Ag processing via the TAP pathway, reflecting the lack of Ag presentation to cytotoxic T lymphocytes (CTLs)[13,46]. Immune function alterations that have been described in HPV infection further include impairment of $CD4^+$ T-cell-mediated immunity and cytokine dysregulation in the blood of women with precancerous lesions or cervical cancer[22,44]. At the systemic level, there is a report that shows a dysregulated CD28 and CTLA-4 expression in peripheral blood T cells of cervical cancer patients, which may lead to impaired function of these lymphocytes and a systemic immunosuppression related to disease progression[47]. A lack of dendritic cells[48,49] and a decrease in helper T cell type 1 immune infiltrates have been described in the presence of HPV-associated lesions[50,51]. Therefore, all these findings provide solid evidence that altered immune responses are a crucial step involved in the carcinogenic events medi-

ated by HPVs.

IL-10 and TGF-β1 cytokine expression in SIL and cervical cancer: Evidence for the generation of local immunosuppression

It is well known that antiviral and anti-tumor immunity in cervical cancer is activated by Th1 cytokines and inhibited by Th2 cytokines. The transformation zone of the cervix, the region most sensitive to SIL and cancer development, is associated with above-average levels of type Ⅱ cytokines (IL-4/IL-6) or immunosuppressive cytokines (IL-10 and TGF-β1) produced by various types of cells, including macrophages, dendritic cells and keratinocytes[52]. Since both cytokines have the ability to interfere with the efficient induction of a type Ⅰ response by antigen-presenting cells (APC), these cytokines may contribute to the predisposition of this region to cervical carcinogenesis[52].

We hypothesize that the changes in the immune response observed during different stages of the HPV infection can support the idea that development of immunosuppressive environment in cervix correlates with the progression of lesions into more aggressive neoplasia. IL-10 (UniprotKB/Swiss-Prot: P22301) is a Th2 anti-inflammatory cytokine that participates in the regulation of the immune response at several levels and can have pleiotropic effects on cell mediators of adaptive and innate immunity[53]. Human IL-10 is produced by CD4+ T cells, activated CD8+ T cells, Epstein-Barr virus-transformed lymphoblastoid cell lines, fibroblasts and monocytes. It has potent inhibitory effects on T cell proliferation and inflammation. IL-10 also depresses the production of a number of Th1 cytokines normally synthesized by activated macrophages and mononuclear cells[53]. More recent studies have clarified that IL-10's immunosuppressive effect on T cells is mainly indirect and is mediated by two other immune cell types: DCs and Treg cells[54]. Additional evidence supports the hypothesis that DCs are the major target of IL-10's immunosuppressive effect. It has been shown that IL-10 reduces the expression of antigen-presenting and costimulatory molecules and interferes with the maturation of monocytes to DCs[55]. These properties support the role of IL-10 as a strong immunosuppressive cytokine and a potent negative regulator of immunoproliferative and inflammatory responses.

A previous study reported a high tendency of IL-10 expression associated with the frequency of HPV types and the severity of the disease[25]. Additionally, Arany *et al*[56] described a mechanism by which IL-10 might enhance persistence and progression of HPV-related lesions under certain conditions (*e.g.*, dysplastic progression, HIV infection) when the cytokine expression in the cervical microenvironment changes. It has been shown that peripheral blood mononuclear cells (PBMC) from patients with both SIL and cervical cancer produce decreased amounts of IL-2 and IFN-γ and higher levels of IL-4 and IL-10 following mitogenic stimulation, compared

with the control group[57]. High levels of IL-10 have been detected in serum and at the cervical level of patients at early stages of the disease[58-60]. The systemic IL-10 mRNA expression level and the IL-10 protein level in serum are significantly higher in SIL compared to women without lesions. The IL-10 expression level is determined by whether a person is a carrier of the allele A of the SNP at -592 nt of the human IL-10 gene[23]. Therefore, the presence of IL-10 appears to be an important factor in the progressive development of the impaired immune response against cervical lesions.

Most cervical tumors expressed IL-4 and IL-10 mRNA and, most importantly, all of them expressed TGF-β1 and IFN-γ mRNA[21,22,61]. IL-10 has been identified by immuno-histochemical analysis in tumor cells and koilocytic cells, but not in tumor-infiltrating lymphocytes, suggesting that the IL-10-producing cells are those transformed by HPV. Similarly, a correlation between immunostaining for IL-10 protein and the level of IL-10 mRNA expression has been reported and supernatant from HPV-transformed cell lines has been found to contain IL-10 and TGF-β1. These findings show a predominant expression of immunosuppressive cytokines, which help down-regulate tumor-specific immune responses in the tumor microenvironment[21,22,61].

A recent study examined T cell functions of PBMC and tumor infiltrating T lymphocytes (TIL) from women with SIL or cervical cancer, including proliferation, cytokine mRNA expression (IL-2, IFN-γ, IL-4, IL-10, TGF-β1), and CD3ζ expression[24]. The distribution of T cells in the epithelium and stroma of biopsies from women with SIL and from women with microinvasive carcinoma of the cervix was determined by immuno-histochemical analysis. There were more TIL in the stroma than in epithelium in advanced stages of the disease where CD8+ T cells prevailed. Consistent with other reports[62], it was found that CD8+ T cells predominate but lack activity compared with CD4+ T cells in women with cervical cancer[24]. To better understand T cell behavior during the course of cervical cancer disease, the proliferation of PBMC and TIL from patients with SIL or cervical cancer with phytohemagglutinin (PHA) or immobilized anti-CD3 was examined and compared to those of healthy donors. For PHA, PBMC from cervical cancer patients proliferated less than those from SIL patients and healthy women, although the differences between SIL patients and healthy women were not significantly different[24]. Moreover, a significant difference was found in anti-CD3-stimulated PBMC between cervical cancer patients and healthy donors. When the proliferation of PBMC *vs* TIL was compared in women with cervical cancer, only PBMC proliferation was statistically higher than that of PHA-stimulated TIL. Thus, women with cervical cancer have a deficient PBMC proliferative response, with no response observed for TIL[24].

Additionally, it has been suggested that reduced T cell function may be associated with alterations in CD3ζ protein expression in cervical cancer patients[63]. A previ-

ous study demonstrated that *in vivo* suppression of CD3ζ chains in patients with CIN can occur as the result of a circulating factor[64]. This circulating factor is composed of IL-10 and TGF-β1, which reduce CD3ζ expression *in vitro*, as show in Figure 1[24]. We therefore propose that IL-10 and TGF-β1 play an important role in generating an immunosuppressive state in the tumor microenvironment which allows the tumor to evade the host cellular immune responses in cervical cancer[24]. A significant correlation between low T cell proliferation and decreased CD3ζ mRNA expression by anti-CD3 stimulated T cells has been reported [24]. Thus, decreased T cell function appears to correlate with cervical cancer progression, which corresponds to a decreased T cell proliferation in cervical cancer patients[24,62,64]. Similarly, a significant positive association between CD3ζ/IL-2 and CD3ζ/IFN-γ expression has been found, indicating that an optimal expression of CD3ζ is associated with the expression of IL-2 and IFN-γ[24,64].

A separate role in immune response escape may be played by TGF-β1 (UniprotKB/Swiss-Prot: P36897), a multifunctional cytokine that prevents cellular immune responses by inhibiting T cell proliferation and differentiation into cytotoxic T cells and helper T lymphocytes. It achieves this by inhibiting stimulatory functions induced by APC. An additional T cell subset, known as Th3, secretes TGF-β1, which can suppress cytotoxic T cell function. Animal and human models suggest that TGF-β1 can promote tumor progression by facilitating extracellular matrix invasion and angiogenesis and by inhibiting immune surveillance[65]. Direct inhibitions of the proliferation and effector functions of CTLs are the immunosuppressive functions of TGF-β1. TGF-β1 may polarize APC activity to favor a Th2 type immune response. In animal models, neutralization of TGF-β1 with monoclonal antibodies or antisense oligonucleotides results in tumor regression or decreased invasiveness[66,67].

After HPV infection of basal epithelia cervical cells, E6 and E7 oncoproteins are expressed (Figure 1), and induce cytokine expression of TGF-β1 throughout the Sp1 transcription factor. The E6-Sp1 and E7-Sp1 complex formation can migrate into the nucleus and induce the TGF-β1 gene expression[68]. TGF-β1 down-regulates IL-2 receptor signaling in T cells and IL-12 expression by APC and induces the expression of IL-10 by macrophages, contributing to immunosuppression during cervical carcinogenesis[68]. Reports have shown increased TGF-β1 expression by cervical cancer, consistent with previous reports of TGF-β1 expression in 94.1% of the stroma surrounding invasive cancer[69]. TGF-β1 gene expression is normally constitutive; however, during tumor progression there is an upregulation of TGF-β1 gene expression, which induces favorable conditions for tumor development[70].

Finally, the presence of IL-10 and TGF-β1 in supernatants from human cervical carcinoma cell lines HeLa and SiHa after 24 h of incubation without FBS has been reported. HPV-positive cervical cell lines produce higher quantities of both cytokines than the HPV-negative C-33A cell line[24]. It was found that the inhibitory effect of rhTGF-β1 is totally counteracted with neutralizing anti-rhTGF-β1 whereas the inhibitory effect of rhIL10 is blocked with neutralizing α-rhIL-10. Thus, both cytokines independently produce a profound inhibitory effect on T cell proliferation[24]. Furthermore, loss of the inhibitory effect of TGF-β1 plays a role in the transformation of normal cervical epithelial cells to dysplastic and malignant cells. Following development of malignancy the TGF-β1 acts to facilitate aggressiveness and tumor progression [71].

Expression of IL-10 and TGF-β1 may be induced by HPV

We hypothesize that the expression of some cytokines, such as IL-10 and TGF-β1, may be induced by HPV, and that IL-10 and TGF-β1 cytokines may be produced by the transformed cell as a mechanism to escape the immune response. Several human heterologous promoters are regulated by HPV proteins. Particularly, HPV-16 E5, E6, and E7 oncoproteins, *trans*-activate a large variety of viral and cellular gene promoters[72]. On the other hand, the presence of HPV E2 regulatory recognition site was reported in the position from -2203 nt to -2191 nt in the IL-10 gene regulatory region[73]. HPV E2 protein is a sequence-specific DNA-binding protein that recognizes the specific sequence ACCN₆GGT present in the viral long control region (LCR) of all HPV genomes[73].

To understand the potential trans-activation ability of HPV E2 protein on the human IL-10 gene expression in cervical cancer, the effects of HPV E2 protein on the promoter activity of human IL-10 gene were evaluated in cervical tumor cell lines, using the luciferase gene reporter assay[74]. The human IL-10 gene regulatory region was obtained by PCR and several construct plasmids that contain different fragments of the IL-10 promoter region were generated in order to transfect HPV-negative C-33A cells. C-33A cells were transfected with the constructs containing the IL-10 regulatory region alone and co-transfected with pCMV16E2 expression plasmid, which expresses HPV E2 protein. For comparison, a plasmid containing the IL-10 complete promoter (pGIL10VB1 plasmid from -2534 nt to +132 nt) and the HPV E2 recognition site was used. The reporter IL-10 gene activity was detected as relative promoter activity, in cells that were transfected with IL-10 complete promoter. This promoter activity increased 6-fold when the cells were co-transfected with pCMV16E2. Similar promoter activity to that of the IL-10 complete promoter was found when the cells were transfected with plasmid that did not contain the HPV E2 recognition site. Thus, we concluded that HPV E2 is able to transactivate human IL-10 gene expression throughout the HPV E2 recognition site into the IL-10 promoter[74].

A synthetic DNA probe containing the HPV E2 recognition sequences present in the IL-10 promoter was designed, and the ability of HPV E2 protein to interact with the E2-binding site was determined using an EMSA assay, to corroborate the effects of the HPV E2 protein
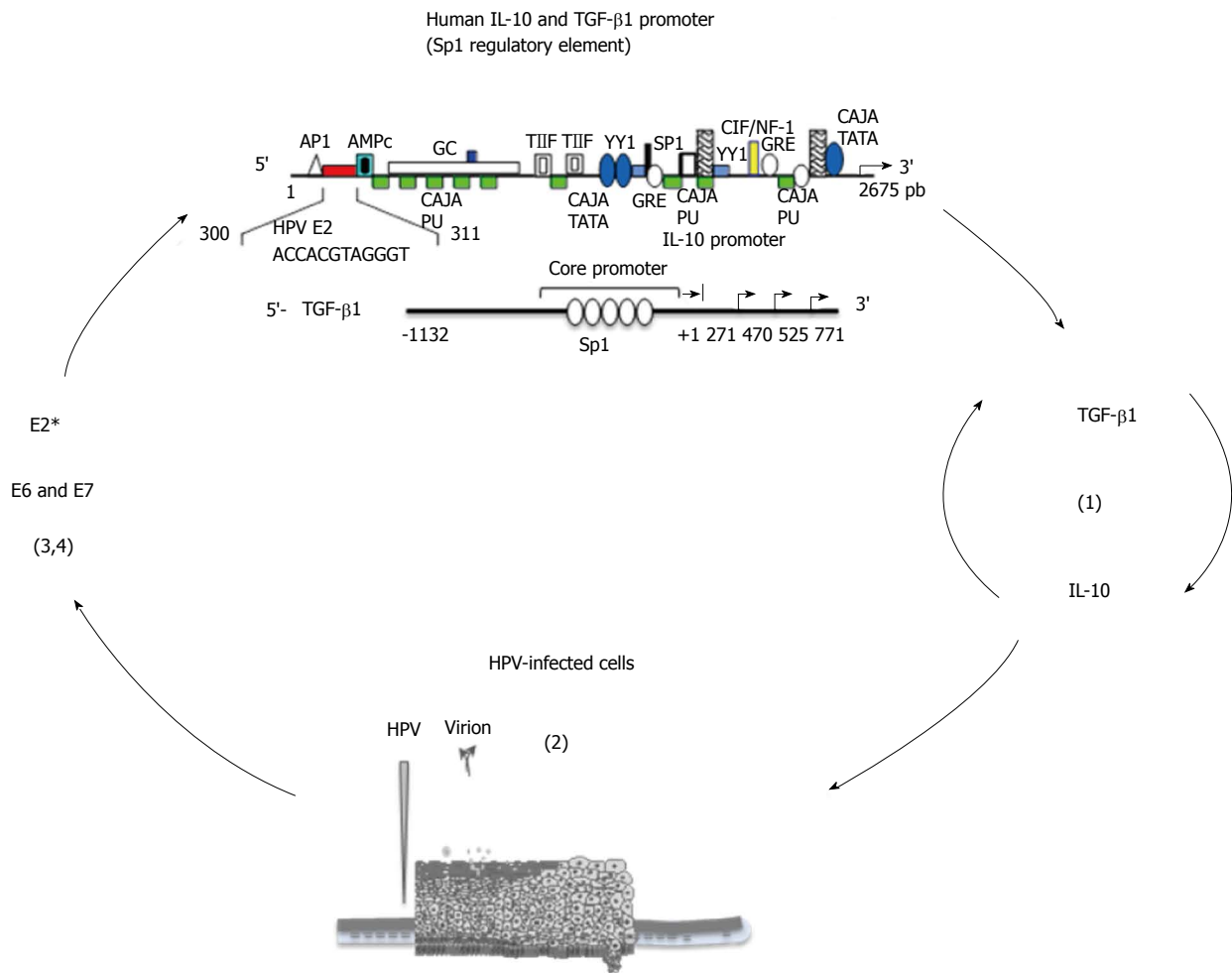
Figure 2 Vicious cycle between Interleukin-10, transforming growth factor-β1, and human papillomavirus. (1) IL-10 enhances TGF-β1 expression and vice-versa[76]; (2) IL-10 induces transcription of HPV-16 E6 and E7[56]; (3) HPV E6 and E7 proteins induce activation of TGF-β1 promoter through Sp1 recognition sequence[21]; (4) Human gene IL-10 regulatory regions posses Sp1 regulatory elements, which suggests that HPV E6 and E7 proteins may bind to IL-10 regulatory region to activate IL-10 gene transcription, just as HPV E2* protein does[74], and may induce IL-10 expression in HPV-transformed cervical cells. HPV can enhance the expression of cytokines TGF-β1 or IL-10, which inhibit T cell functions and augment viral proteins, contributing to the persistence of HPV infection and progression of HPV-related cervix lesions to cervical cancer. The production and activity of these two cytokines, IL-10 and TGF-β1, are interrelated and likely involve a positive feed-back loop in which IL-10 enhances the expression of TGF-β1 and vice-versa[76]. In fact, IL-10 not only enhances the production of TGF-β1 but also controls the ability of target cells to respond to TGF-β1. This involves the IL-10-mediated restoration of the expression of TGF-β1 receptor 2 in recently activated T cells, which usually down-regulate this receptor and become desensitized to the inhibitory effects of TGF-β1. Conversely, TGF-β1 can promote the production of IL-10[76]. IL-10: Interleukin-10; TGF-β1: Transforming growth factor-β1; HPV: Human papillomavirus.

on the transactivation mechanism of the IL-10 promoter and the importance of physical interaction with the HPV E2 recognition site. HPV E2 protein is able to bind to the IL-10 gene promoter, particularly with in the HPV E2 recognition site. The evaluation of IL-10 gene expression in HPV E2 transfected cells, measuring IL-10 mRNA by semiquantitative RT-PCR analysis, demonstrated that HPV E2 is able to induce IL-10 gene expression. This evidence contributes to the knowledge of the IL-10 gene expression molecular mechanism induced by HPV E2 protein, and is crucial to understanding the role of IL-10 in the process of HPV cervical carcinogenesis[74].

These results lead us to propose a novel molecular pathway through which HPV E2 protein may stimulate IL-10 gene expression. This finding suggests a potential mechanism through which HPV proteins regulate IL-10

gene expression during cervical cancer development, which may represent an immune response regulation strategy mediated by HPV proteins[74]. Additionally, it has been demonstrated that IL-10 induces transcription of the E7 early promoter, through the segment of the upstream regulatory region[56]. Also, cervical keratinocytes express IL-10 receptor mRNA, which may explain IL-10's autocrine activity on tumour cells and HPV transcription in a dose-dependent manner. IL-10 expression by cervical cells after HPV infection is associated with enhanced viral persistence and progression of HPV- lesions to cancer[75].

To gain insight into the potential TGF-β1 gene regulation mechanism produced by the high-risk HPV E6 and E7 oncoproteins, the TGF-β1 promoter activity induced by these oncoproteins was analyzed. When the TGF-β1

core promoter in HPV E6- and E7-expressed tumor cells was evaluated, an increase in the reporter gene expression was found, compared with E6 and E7 non-expressed tumor cells. The TGF-β1 promoter *trans*-activation was slightly elevated in E7 compared to E6 expression cells. These findings support the notion that the TGF-β1 promoter activation by HPV-16 E6 and E7 could be physiologically relevant, particularly in cell transformation and immortalization[68].

A cluster of five DNA binding motifs 100% homologous to Sp1 recognition sites was found in the TGF-β1 core promoter sequence (-650 nt to +36 nt). DNase Footprinting assays *in vitro* were used to examine the profile of DNA-protein interaction at the TGF-β1 core promoter to identify the target sequences responsible for TGF-β1 promoter *trans*-activation by HPV-16 E6 and E7 oncoproteins. A differential protection pattern in the TGF-β1 core promoter was found which suggests that HPV-16 E6 and E7 oncoproteins may induce varied interactions in tumor cells and may favor the recruitment of co-activators and/or transcription factors. This finding indicates that HPV-16 E6 and E7 oncoproteins expression may induce the formation of novel molecular transcription complexes throughout Sp1 recognition sites in tumor cells during cervical cancer development[68].

In addition, the *trans*-activation caused by E6 and E7 can be abolished by mutation in the Sp1e (-108 to -102) recognition site. The data shows that HPV-16 E6 and E7 oncoproteins bind first to the Sp1 transcription factor and then the Sp1-E6 or Sp1-E7 complex binds to the TGF-β1 regulatory element site (GGGGCGG). They do not bind directly to DNA[68]. The physical interactions and functional cooperation between HPV E6 and E7 oncoproteins and cellular regulatory elements at the TGF-β1 promoter explain the contribution of HPV-16 to TGF-β1 gene expression in cervical cancer[68]. Similarly, human gene IL-10 regulatory region possesses this Sp1 regulatory element (GGGGCGG). This suggests that HPV E6 and E7 proteins may bind to the IL-10 regulatory region to activate IL-10 gene transcription, just as the HPV E2 protein does, which may induce IL-10 expression in cervical HPV-transformed cells, as shown Figure 2.

CONCLUSION

The presence of IL-10 and TGF-β1 in high-risk HPV cervical infections and patients with SIL may constitute an early event that promotes a microenvironment in the lesion with negative effects on the cellular immune response. Such a microenvironment could favor virus persistence and progression to cervical cancer. These cytokines have been detected in serum and cervical tissues from patients with high-risk HPV infection, with low grade SIL, high grade SIL, and cervical cancer. Their levels increase in correlation with the severity of the lesions[25]. Results discussed in this review suggest that cervical cancer is characterized by local immunosuppression

dependent on Th2/Th3 cytokines. This data is in agreement with findings in cervical biopsies in which there is a pattern of Th2/Th3 cytokine expression present in cervical cancer but absent in normal cervix, suggesting that HPV infection induces the transcription of immunosuppressive cytokines as a means of evading the host immune system[21,22,61].

IL-10 is a potent immunosuppressive cytokine that induces and is induced by TGF-β1 expression. It also induces the HPV-16 E6 and E7 proteins, which induce TGF-β1 transcription activation and IL-10 gene expression to create a vicious cycle. IL-10 and TGF-β1 also down regulate CD3ζ expression, which plays a crucial role in T-cell activation. Finally, IL-10 and TGF-β1 induce the recruitment of Treg cells, which produce a profound peripheral tolerance. In summary, we postulate that IL-10 and TGF-β1 induce immune system evasion through an immunosuppressive state in the environment of the cervix in women infected with HPV. This information is highly relevant in the areas of HPV vaccine generation and the design of new targeted immunotherapies for women with LGSIL, HGSIL, and cervical cancer[77].

The future of research on the immune system in the context of HPV-associated cervical cancer has more questions than answers. Since it has been demonstrated that T-lymphocytes from patients with cervical lesions and cervical cancer are partially activated and had reduced expression of several signal transduction molecules which are involved in the complete activation of T-lymphocyte, it is of great interest to investigate whether the expression of these molecules can be reversed by type Th1 cytokines and whether they can turn these HPV-specific T-lymphocytes fully functional.

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