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HPV16 E2 protein promotes innate immunity by modulating immunosuppressive status

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ABSTRACT

The balance between active immune responses against human papillomavirus (HPV) and HPV-induced immune escape regulates viral clearance and carcinogenesis. To understand the role of the early viral protein HPV16 E2 in host innate immune responses, the HPV16 E2-transfected murine squamous cell carcinoma cell line SCCVII (SCC/E2) was generated and anti-tumor responses in T-cell-depleted mice were evaluated. Tumor growth of SCC/E2 was markedly reduced. Cytotoxicity against the NK-sensitive targets YAC-1 and SCCVII was clearly enhanced in SCC/E2-inoculated mice. Despite the comparable ratio of NK cells, the proportion of $CD11b^{+}Gr\text{-}1^{+}$ myeloid-derived suppressor cells (MDSCs) was significantly decreased in SCC/E2-inoculated mice. The transcription of MDSC-related mediators such as inducible nitric oxide synthase, indoleamine 2,3-dioxygenase, and heme oxygenase-1 was significantly impaired in the SCC/E2-inoculated tumor tissues on day 3. Our results suggest that HPV16 E2 promotes anti-tumor innate effector function by modulating immunoregulatory events mediated by MDSCs and their mediators. This report describes a new role for HPV16 E2 as a local immunomodulator at infected sites.

1. Introduction

Human papillomavirus 16 (HPV16) is most frequently found in cervical cancer. More than half of females show cervical HPV infections soon after they become sexually active, but about 90% of such infections are cleared within 2–3 years while the remainder form a persistent infection and result in malignant transformation. Thus, the balance between immune responses against HPV and HPV-induced immune escape is important for viral clearance and carcinogenesis [1,2].

The circular DNA genome of HPV contains an early region, encoding the early proteins E6, E7, E8, E1, E2, E4, and E5, and a late region, encoding the viral capsid proteins L1 and L2 [2]. These viral proteins are differentially expressed depending on the cell differentiation program. The long control region (LCR) is a non-encoding region involved in replication and viral transcription. HPV infection requires viral particles to move across the basal layer of the cervical epithelium. The viral proteins E1 and E2, which are initially expressed in basal epithelial cells, regulate viral replication and transcription. E2 binds to the four E2-binding domains in the LCR and regulates the transcriptional levels of the viral oncogenes E6 and E7. When E2 expression is lost, the HPV genome integrates into the host's DNA.

During the early stages of an HPV infection, the host immune response becomes the first line of defense against the infection. In cooperation with keratinocytes (KC), dendritic cells and natural killer (NK) cells promote a cytokine or chemokine-mediated proinflammatory process, which links innate and adaptive immune responses. HPV as a dsDNA virus may be recognized using intracellular pattern recognition receptors such as Toll-like receptor (TLR) 9 in KC, leading to the secretion of cytokines. Type-1 interferons (IFNs) and interleukin (IL)-12 are crucial cytokines that inhibit viral replication and activate NK cells [3]. NK cells are the most potent early immune effector cells that kill HPV-infected KC [4]. However, HPV, especially the oncoproteins E6 and E7, exert various mechanisms of immune evasion such as the down-regulation of type-1 IFNs and their related signaling [2,5,6] and the inhibition of a panel of cytokines and chemokines in KC [4]. HPV16-specific T-cell responses were detected in half of patients with $HPV16^{+}$ low-grade

Abbreviations: KC, keratinocytes; MDSC, myeloid-derived suppressor cells; IDO, indoleamine 2,3-dioxygenase; HO-1, heme oxygenase-1; iNOS, inducible nitric oxide synthase; CIN, cervical intraepithelial neoplasia.

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squamous intraepithelial lesions [7]. The presence of E2-specific T-cell responses was correlated with the regression of HPV16$^+$ lesions, while the detection of E6-specific reactivity was associated with persistence. Recombinant HPV16 E2 has been shown to promote the regression of HPV-transformed human epithelial tumors in immunocompetent nude mice [8] and of already established HPV16-transformed mouse tumors in immunoincompetent mice, suggesting the active anti-tumor effects of HPV16 E2. HPV16 E2 has been shown to exert pro-apoptotic activity in both human and murine HPV16-transformed epithelial cells [9,10]. Therefore, it is difficult to determine whether tumor regression is the result of host immune responses or the induction of apoptosis by HPV16 E2, which functions as a viral repressor of HPV E6/E7 transcription. In this study, we generated HPV16 E2-transduced murine epithelial squamous cell carcinoma (SCC) cells that grew at the same speed as parental SCC cells and examined the roles of HPV16 E2 in host innate immune responses by intradermal transplantation in T-cell-depleted mice.

2. Materials and methods

2.1. Mice

Female 6-week-old C3H/HeN (C3H) mice were purchased from Japan SLC (Hamamatsu, Japan). Mice were maintained under specific pathogen-free conditions. All procedures were reviewed and approved by the Animal Care and Use Committee of the Tokyo Medical and Dental University.

2.2. HPV16E2 vector construction and transfection

The vector containing HPV16 E2 (pCMV4-HPV16E2) was kindly provided by Dr. Alison McBride, NIH (Bethesda, MD). HPV16 E2 was subcloned into a pIRES2-EGFP expression vector with *Sac*I and *Sma*I sites, after which the inserted sequence was confirmed. The SCC cell line SCCVII (C3H-originated) was transfected with either HPV16 E2/pIRES2-EGFP or mock-pIRES2-EFGP plasmid using Lipofectamine 2000. After drug selection, transfectants expressing high levels of GFP were sorted by flow cytometry, as described previously [11,12]. Two HPV16 E2/GFP-transfectants (SCC/E2.1 and SCC/E2.2) and a mock/GFP-transfectant (SCC/GFP) were used.

2.3. In vitro stimulation of SCC cells

Cells (1×10^4/ml) were stimulated with a TLR9 ligand, CpG (3 μM, CpG-B-ODN, Type K; Hycult Biotech, Plymouth Meeting, PA) in a 24-well plate for 24 h. Cells were harvested for flow cytometry and total RNA isolation.

2.4. Monoclonal antibodies (mAbs) and flow cytometry

mAbs against mouse H-2K^k (36-7-5, mouse IgG2a), retinoic acid early inducible-1 (RAE-1γ) (CX1, rat IgG2b), murine UL16-binding protein-like transcript (MULT)1 (5D10, Armenian hamster IgG), CD49b (DX5, rat IgM), CD3 (145-2C11, hamster IgG), CD45R (RA3-6B2, rat IgG2a), Gr-1 (Ly6C/Ly6G, RB6-8C5, rat IgG2b), and CD11b (M1/70, rat IgG2b) were used. All FITC, PE-, allophycocyanin (APC)-conjugated and biotinylated mAbs and isotype control IgG antibodies were obtained from eBioscience (San Diego, CA) or BD Pharmingen (San Diego, CA). Culture supernatant from the 2.4G2 hybridoma (anti-CD16/CD32 mAb) was used to block nonspecific binding. Stained cells were analyzed using BD FACSCalibur and CellQuest software (BD Biosciences, San Jose, CA).

2.5. RT-PCR and quantitative real-time PCR

Total RNA was extracted from cultured cells or tumor-inoculated tissues using Nucleospin RNA II (Takara Bio, Tokyo). First-strand cDNA was synthesized using oligo(dT) primers and reverse-transcribed using Primescript RT Master Mix (Takara Bio). RT-PCR was performed using GFP, HPV16E2, and GAPDH primer sets. cDNA was amplified on a DNA thermal cycler (Perkin Elmer, Waltham, MA) for 40 cycles (95 °C for 5 min, 95 °C for 30 s, 58 °C for 15 s, and 72 °C for 30 s) followed by a 7-min extension at 72 °C. Real-time PCR was performed using a LightCycler instrument and DNA Master SYBR Green I kit (Roche Diagnostics, Indianapolis, IN). The primer sequences used in this study are shown in Supplemental Table. The data were evaluated using the LightCycler software and are presented as the relative ratio of the respective molecule against GAPDH.

2.6. Tumor inoculation

To deplete T cells in C3H mice, 0.5 mg each of anti-CD4 (GK1.5) and anti-CD8 (53.6.72) mAbs were intraperitoneally administrated on days -4, -1, 3, and 7 [12,13]. The depletion of T cells less than 5% in splenocytes and 1% in peripheral blood lymphocytes was confirmed by flow cytometry at day 12. Parental or transfected SCC cells (3×10^5/mouse) were intradermally inoculated into the shaved right flank of C3H mice and tumor volumes were evaluated

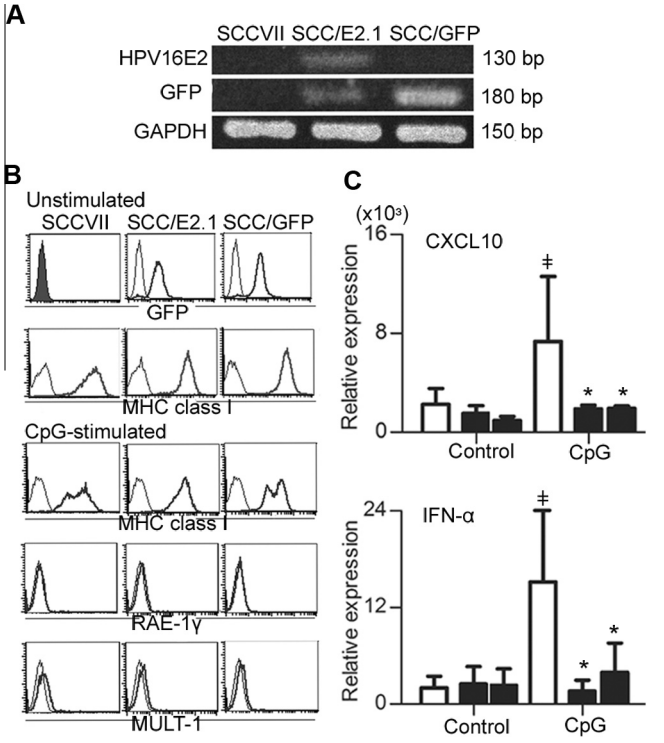

Fig. 1. Characterization of HPV16 E2-transfected SCCVII cells. (A) Total RNA was extracted from SCCVII, HPV16 E2/GFP-transfected SCCVII (SCC/E2.1), or GFP-transfected SCCVII (SCC/GFP) cells and was then reverse-transcribed and amplified by PCR. (B) GFP expression in SCCVII, SCC/E2.1, and SCC/GFP cells was analyzed by flow cytometry. The data are displayed as histograms (4-decade logarithm scales) with the control histograms nearest to the ordinate (plain lines). The results for SCC/E2.2 were similar to those for SCC/E2.1 in A and B. (C) Total RNA was extracted from unstimulated or CpG-stimulated SCCVII (open bars) or SCC/E2.1 and SCC/E2.2 (black bars) cells, and real-time PCR to amplify CXCL10 and IFN-α was performed. The values represent the means ± SD from each group of three samples, and the relative expression level, as compared to that of GAPDH. *Significantly different from unstimulated SCCVII ($^‡p < 0.05$) or CpG-stimulated SCCVII ($^*p < 0.05$) cells.

[13]. Tumor and lymphoid tissues were obtained on day 3 and used for cytotoxicity assays and real-time PCR.

2.7. B-cell depletion and cytotoxicity assay

In T-cell-depleted mice, B cells are the major splenocytes; therefore, we used B-cell-depleted splenocytes for a cytotoxicity assay. B cells were depleted using biotinylated anti-CD45R mAb streptavidin particles plus-DM and the BD IMag separation system (BD Biosciences). B cell depletion to less than 5% of the $CD45R^{+}CD3^{-}$ cells in each sample was confirmed by flow cytometry. B-cell-depleted splenocytes were used as effector cells. Cytotoxicity against YAC-1 (an NK-sensitive T-lymphoma cell line), SCCVII, and SCC/E2.1 was measured using a CytoTox96 Non-Radioactive Cytotoxicity Assay Kit (Promega, Madison, WI) according to the manufacturer's protocol.

2.8. Statistical analysis

Statistical analyses were performed by a one-way analysis of variance or the Mann–Whitney *U*-test using SPSS or GraphPad Prism (San Diego, CA) software. Values of $p < 0.05$ were considered to indicate significance.

3. Results and discussion

3.1. HPV16 E2-introduction into murine SCC cells alters chemokine expression

The mRNA and protein levels of HPV16 E2 and GFP in SCCVII cells that had been transfected with either HPV16 E2/GFP or GFP alone were confirmed by RT-PCR and flow cytometry. SCC/E2 alone showed a specific HPV16 E2 mRNA band (Fig. 1A), and the cell surface protein levels of GFP were similar to those of SCC/E2 and SCC/GFP (Fig. 1B, top panels). The expression levels of MHC class I molecules, which contribute to NK-mediated killing, were comparable, and stimulation with CpG as a TLR9 ligand down-regulated MHC class I expression to roughly the same extent in all three types of cells. Two NKG2D ligands, RAE-1γ and MULT1, were not substantially expressed in either unstimulated (data not shown) or stimulated cells (Fig. 1B).

Our preliminary microarray analyses suggested that HPV16 E2 regulates various cytokine/chemokine genes in human KC. The expression of CXCL10 (IFN-γ-induced protein, IP-10), which promotes the recruitment of macrophages and NK cells to inflamed tissues [14], and of IFN-α, which is a critical cytokine for virus elimination [3], in SCCVII cells was markedly elevated by CpG

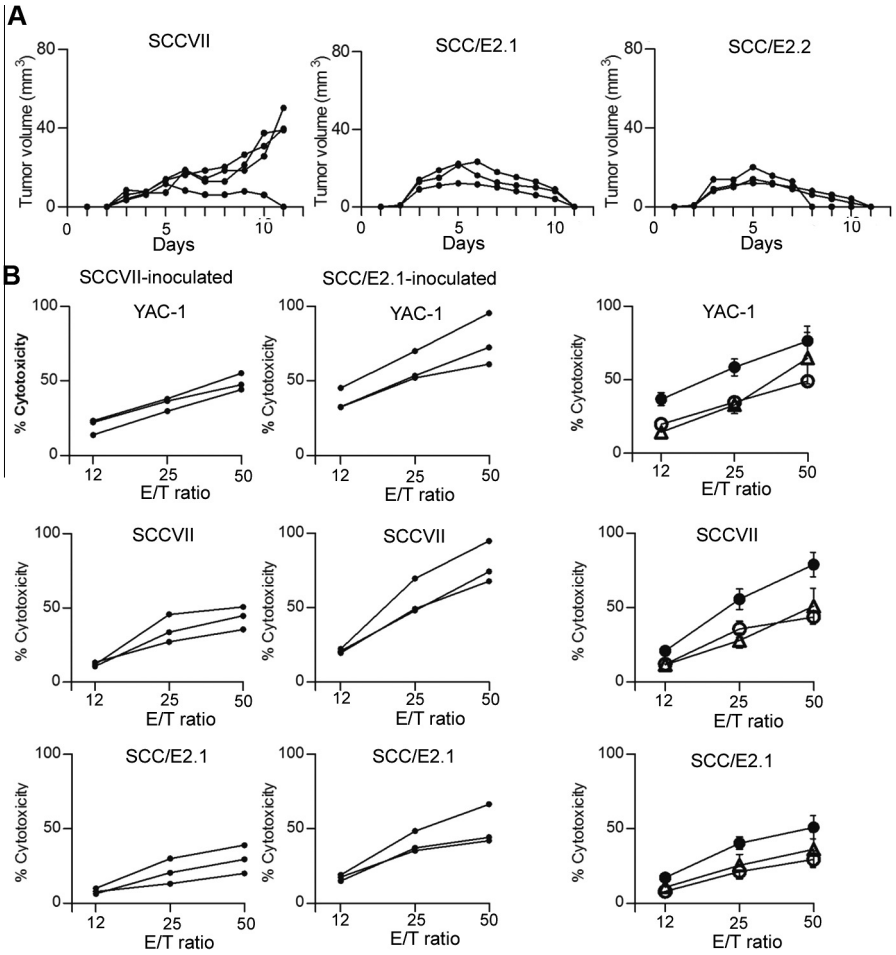

Fig. 2. *In vivo* anti-tumor responses and *in vitro* cytolytic effector function were augmented in SCC/E2-inoculated mice. (A) SCCVII, SCC/E2.1, and SCC/E2.2 cells (3×10^5) were injected intradermally into T-cell-depleted C3H mice, after which the tumor volume was monitored. The data are representative of two independent experiments. (B) Splenocytes were collected at 3 days post-tumor inoculation of SCCVII, SCC/E2.1, and SCC/GFP. B-cell-depleted splenocytes were isolated. The B-cell-depleted splenocytes still contained 5–8% $CD45R^{+}$ B cells and 0.1–1.5% $CD3^{+}$ T cells, and no differences in these cell populations were observed between the experimental groups. The cytotoxicity of B-cell-depleted splenocytes against YAC-1, SCCVII, and SCC/E2.1 was measured by an LDH releasing assay. The level of cytotoxicity in individual mice from the SCCVII- and SCC/E2.1-inoculated groups and the mean values from the SCCVII-(open circles), SCC/E2.1-(closed circles), and SCC/GFP-(triangles)-inoculated groups (three mice per group) are shown. Cytotoxicity of B-cell-depleted splenocytes against YAC-1 from *in vivo* T-cell-depleted and tumor non-inoculated mice was 9.0 ± 4.0%, 10.7 ± 4.9%, and 21.1 ± 2.9% at the E/T ratio of 12.5, 25 and 50, respectively.

stimulation, but their expression in two SCC/E2-transfectants was clearly impaired (Fig. 1C). Our results suggest that SCC/E2 introduction into murine SCC cells inhibited the TLR9 signaling-induced secretion of innate cell-attracting chemokines and antiviral cytokines.

3.2. HPV16 E2 enhances anti-tumor responses and cytolytic effector function

HPV initially infects epithelial KC, from which cervical cancer originates. To mimic infected KC, parental SCCVII or SCC/E2 transfectants were intradermally administrated in T-cell-depleted syngeneic C3H mice and the tumor volumes were monitored. The tumor volume in the SCCVII-inoculated mice increased gradually until day 11, except for one mouse that showed regression after day 5. In contrast, the tumors in the SCC/E2-inoculated mice regressed after the initial tumor growth until day 5. All of the inoculated SCC/E2 tumors were completely eliminated by day 11 (Fig. 2A). There was no clear difference in cell growth between SCCVII and SCC/E2 cells in culture (data not shown). These results indicate that HPV16 E2 introduction reduced the tumorigenicity of SCCVII cells in T-cell-depleted host mice.

To investigate whether the reduced tumorigenicity caused by HPV E2 introduction was mediated by host immune responses, we first examined the status of splenocytes from tumor-inoculated mice. The proportion of NK cells and NK-mediated cytotoxicity were evaluated using B-cell-depleted splenocytes at 3 days post-tumor inoculation, since the mean tumor volume at this time point was comparable. The percentage of $CD3^{-}CD49b^{+}$ NK cells was 30.5 ± 1.2% and 32.6 ± 1.7% (mean ± SD) in the SCCVII- and SCC/E2-inoculated mice, respectively, and no clear difference was observed between the groups. The SCC/E2-inoculated mice showed consistently greater cytotoxicity against all three targets, as compared to the SCCVII-inoculated mice (Fig. 2B). It should be noted that both groups of splenocytes showed lower cytotoxicity against SCC/E2, as compared with parental SCCVII, suggesting that HPV16 E2 transduction enhances NK resistibility rather than NK sensitivity. B-cell-depleted splenocytes from the SCC/GFP-inoculated mice showed comparable cytotoxicity against all three targets to those from SCCVII-inoculated mice (Fig. 2B, right panels) and comparable NK cell ratios (data not shown), suggesting that the enhanced cytotoxicity was unrelated to GFP expression. Our results indicate that HPV16 E2 introduction to target cells induces NK resistibility at the effector phase. It appears that the enhancement of NK cell-mediated cytotoxicity in HPV16 E2 at the induction phase *in vivo* is mediated by another mechanism.

3.3. HPV16 E2 reduces immunoregulatory status

$CD11b^{+}Gr\text{-}1^{+}$ myeloid-derived suppressor cells (MDSCs) are known to modulate infection and anti-tumor responses. Murine MDSCs may be further divided into granulocytic and monocytic MDSCs based on their level of Ly6C expression [15–18]. Recent reports have shown that MDSCs regulate the generation and function of NK cells and that $CD11b^{high}CD27^{high}$ NK cells were converted into MDSCs in the tumor microenvironment [19–21]. We evaluated $Gr\text{-}1^{+++}CD11b^{++\sim+++}$ cells as $Ly6C^{low}$ granulocytic MDSCs (Gr-MDSCs) and $Gr\text{-}1^{++}CD11b^{++}$ cells as $Ly6C^{high}$ monocytic-MDSCs (Mo-MDSCs) (Fig. 3A), since a preliminary analysis using anti-Ly6C mAbs showed differential expression levels of Gr-1. Interestingly, the proportion of Gr-MDSCs was significantly decreased in SCC/E2-inoculated mice (Fig. 2B). The reciprocal reduction in the MDSC ratio and enhancement of NK-mediated cytotoxicity in SCC/E2-inoculated splenocytes suggests a link between these two phenomena. To demonstrate a reduction in MDSCs in tumor tissues, we performed immunohistochemical analyses with anti-CD11b and -Gr-1 mAbs, but we could not clearly evaluate the differences because $CD11b^{+}$ signals were observed in most cells (e.g., infiltrating cells, stromal cells, and tumor cells) and the Gr-1 signals were too weak to allow a reliable quantitative evaluation. Since the tumor at this time point was very small, we were unable to isolate enough tumor-infiltrating cells for the direct determination of MDSC and NK cell recruitment by flow cytometry.

The tumor microenvironment contains an array of immunoregulatory molecules. The suppressive activity of MDSCs is associated with L-arginine metabolism. L-arginine serves as a substrate for two enzymes, inducible nitric oxide synthase (iNOS), which generates NO, and arginase 1 (Arg1) [15]. The tryptophan catabolic enzyme indoleamine 2,3-dioxygenase (IDO, IDO-1) plays an important role in immune regulation [22,23]. Heme oxygenase-1 (HO-1), which is overexpressed in the tumor microenvironment, regulates oxidative stress and inhibits active immune responses [24,25]. MDSCs are known to produce large amounts of HO-1 [26]. We examined the transcriptional expression of MDSC-related regulatory molecules in the tumor tissues on day 3. The expression of

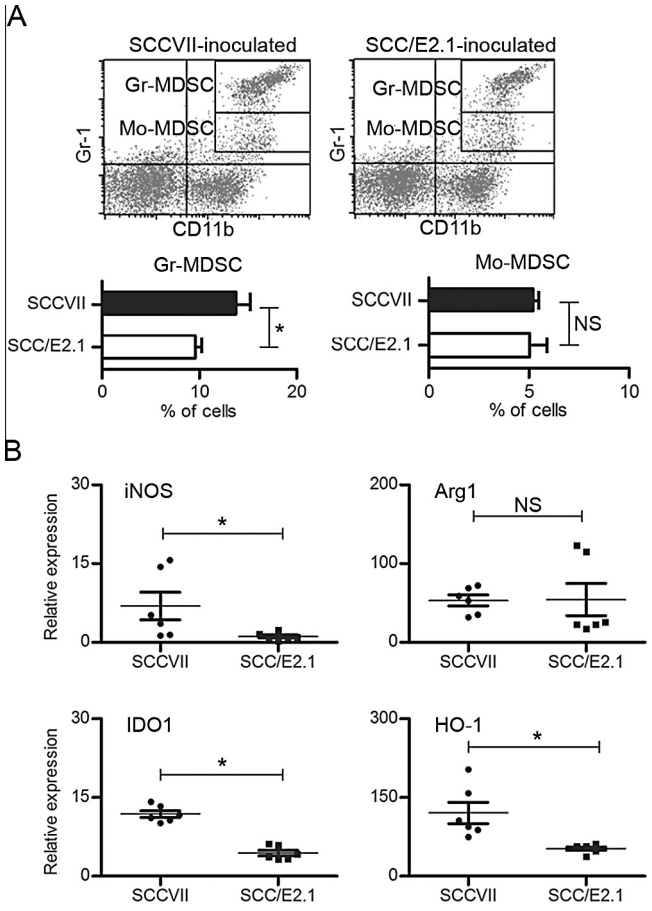

Fig. 3. HPV16 E2 reduces immunoregulatory status. (A) B-cell-depleted splenocytes were obtained as described in Fig. 2. Cells were stained with FITC-conjugated anti-Gr-1 and APC-conjugated anti-CD11b mAbs and then analyzed by flow cytometry. An electronic gate was placed on the large lymphocyte gate, including myeloid cells and granulocytes, after which expression profiles for Gr-1 and CD11b were displayed as dotted plots. A representative profile from each group is shown. Two regions for $Gr\text{-}1^{+++}CD11b^{++\sim+++}$ cells and $Gr\text{-}1^{++}CD11b^{++}$ cells were placed and assessed as granulocytic-MDSCs (Gr-MDSCs) and monocytic-MDSCs (Mo-MDSCs), respectively. The values represent the means ± SD from each group of three mice. *Significantly different ($p < 0.05$). The data are representative of two independent experiments. (B) Tissues from SCCVII- or SCC/E2-inoculated sites were resected and total RNA was extracted. The expression of the indicated molecules was then evaluated. Each symbol presents individual values. Bars show the mean ± SD from each group of seven mice, and the relative expression levels against that of GAPDH are shown. *Significantly different ($p < 0.05$).

iNOS, IDO, and HO-1 was significantly impaired in the SCC/E2-inoculated tissues (Fig. 3C). Although CpG-stimulated SCC/E2 increased CXCL10 and IL-1α expression *in vitro* (Fig. 1C), we did not observe clear differences in chemokine/cytokine expression in the tumor-inoculated tissues (CXCL10, CCL5, CCL2, CXCL2, IFN-α, IFN-γ, IL-1α, TNF-α, TGF-β, and IL-10) (data not shown). In addition to tumor cells, epithelial KC, Langerhans cells, stromal cells, and tissue-infiltrating immune cells may secrete various inflammatory mediators, which positively and negatively regulate local immune responses and inflammation.

In this study, we demonstrated that HPV16 E2 introduction into murine SCC cells augmented T-cell-independent early anti-tumor immune responses. We also found that the inoculation of SCC/E2 enhanced cytolytic effector function, and reversely inhibited the proportion of MDSCs in the spleen and the local expression of MDSC-related immunoregulatory mediators in tumor tissues. Our results suggest that HPV16 E2 reduces the immunoregulatory status in the local microenvironment, which augments tumor-eradicating innate immune responses, presumably mediated by NK cells.

Consistent with our observations, a recombinant adenovirus inducing expression of HPV16 E2 was shown to exhibit potent anti-tumor efficacy in cervical cancer by local delivery [27]. Clinical trials have been performed to evaluate the use of modified vaccinia virus Anlara expressing the E2 gene of bovine papillomavirus (MVA E2) to treat cervical intraneoplasia (e.g., cervical intraepithelial neoplasia [CIN] 1 or CIN 2) and CIN 3 lesions associated with HPV infection [28,29], as well as intraurethral flat condyloma in men [30]. Therapeutic vaccination has been shown to effectively induce the production of antibodies against E2 and cytotoxicity against HPV-transformed tumor cells. However, the contribution of HPV16 E2 to early inflammatory responses was not addressed in these studies. In this report, we shed light on the immunomodulatory effects of HPV16 E2 as a repressor of MDSCs and MDSC-related mediators. It has been shown that IDO expression levels are higher in cervical cancer than in normal cervix, in CIN, [31] and in HPV16 E7-transduced skin [32]. The inhibition of IDO reversed local immune suppression and inhibited tumor growth by promoting NK cell accumulation and cytotoxicity [32,33]. Although SCC cells preferentially induce the accumulation of MDSCs, recent data indicate that the ligand-mediated activation of TLR signaling in the epithelium also induces immune suppression, and MDSCs are a major negative feedback mechanism for controlling excess tissue damage triggered by pathogens [34]. Our results indicate a new role for HPV16 E2 and provide insight into therapeutic applications of the early protein E2.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.03.042.

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