CANCER IMMUNOLOGY RESEARCH | RESEARCH ARTICLE

Myeloid Cells Orchestrate Systemic Immunosuppression, Impairing the Efficacy of Immunotherapy against HPV+ Cancers

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ABSTRACT

Cancers induced by human papillomaviruses (HPV) should be responsive to immunotherapy by virtue of expressing the immunogenic oncoproteins E6/E7. However, advanced forms of cervical cancer, driven by HPV, are poorly responsive to immune response–enhancing treatments involving therapeutic vaccination against these viral neoantigens. Leveraging a transgenic mouse model of HPV-derived cancers, K14HPV16/H2b, we demonstrated that a potent nanoparticle-based E7 vaccine, but not a conventional “liquid” vaccine, induced E7 tumor antigen–specific CD8+ T cells in cervical tumor–bearing mice. Vaccination alone or in combination with anti-PD-1/anti-CTLA4 did not elicit tumor regression nor increase CD8+ T cells in the tumor microenvironment (TME), suggesting the presence of immune-suppressive barriers. Patients with cervical cancer have poor dendritic cell functions, have weak cytotoxic lymphocyte responses, and demonstrate an accumulation of myeloid cells in the periphery. Here, we illustrated that myeloid cells in K14HPV16/H2b mice possess potent immunosuppressive activity toward antigen-presenting cells and CD8+ T cells, dampening antitumor immunity. These immune-inhibitory effects inhibited synergistic effects of combining our oncoprotein vaccine with immune checkpoint–blocking antibodies. Our data highlighted a link between HPV-induced cancers, systemic amplification of myeloid cells, and the detrimental effects of myeloid cells on CD8+ T-cell activation and recruitment into the TME. These results established immunosuppressive myeloid cells in lymphoid organs as an HPV+ cancer--induced means of circumventing tumor immunity that will require targeted abrogation to enable the induction of efficacious antitumor immune responses.

Introduction

Oncogenic human papillomaviruses (HPV) are the etiologic cause of various malignancies, including cervical cancer and oral/head and neck cancer (1). Cancer progression in infected cells is driven by the viral proteins E6 and E7, which interfere with the activity of the p53 and pRB tumor suppressors (2, 3), eventually leading to malignancy. The vast majority of HPV infections are eradicated by the immune system (4); however, in a small percentage of infected individuals the virus persists and elicits premalignant proliferative lesions, intraepithelial neoplasias (5), that, if not eliminated by a natural immune response or therapeutic intervention, can evolve to invasive squamous cell carcinomas (6–8). Immunotherapy has taken center stage in cancer treatment (9), and immune checkpoint–blocking antibodies are regularly receiving approval for new types of cancer (10). However, many patients do not respond to such therapies (11), thus efforts are refocusing on understanding the underlying innate and acquired resistance mechanisms to immunotherapies (10, 12). Another effort is focused on neoantigen vaccines designed to stimulate tumor-specific immune responses, including leveraging the E7 oncoprotein for HPV-induced malignancies (13–17). Surprisingly, approaches based on immunization with E7 peptides have shown only modest success as monotherapy against invasive cancers (14, 16). Combinations with immune checkpoint blockade (ICB) have shown more encouraging results, but the percentage of responding patients remains low (17).

Patients with cervical cancer exhibit systemic alterations in their immune system, beyond the tumor microenvironment (TME), including low numbers of circulating dendritic cells (DC), poor antigen-presenting cell (APC) function, weak cytotoxic lymphocyte (CTL) responses, and the accumulation of myeloid cells in the periphery (18–22). Collectively, these deficiencies may contribute to the poor efficacy of immunotherapy-based treatments, including therapeutic vaccination, against cervical carcinomas. Although some reports suggest that myeloid cells might be playing an immunosuppressive role in this disease (20, 22), their activity remains poorly characterized following immunotherapy in the setting of HPV-related cancers. The mechanisms responsible for the various immune-related defects observed in patients with HPV-induced malignancies remain obscure.

Here we showed that a genetically engineered mouse model (GEMM) of HPV-induced malignancies, K14HPV16/H2b,
recapitulated the aforementioned alterations in immune cell functions and defects in antitumor immune responses. This enabled the investigation of mechanism underlying immunosuppression manifested by an overaccumulation of myeloid cells in lymphoid organs.

**Materials and Methods**

*Mice, tumor cells, and antibody treatments*

K14HPV16/H2b mice or their FVBN/H2b littermates were generated at University of California San Francisco (San Francisco, CA) in the Hanahan lab. A preliminary characterization of the K14HPV16/H2b model was reported previously (23) now substantiated by further analyses presented here. The K14HPV16/H2b mouse line was generated by crossing K14HPV16/FVB (H2q) mice (24, 25) with C57BL/6 (H2b) mice to introduce the entire H2b locus, followed by backcrossing to FVB to render the mice congenic for H2b but otherwise genetically FVBN. F1 mice were backcrossed for 11 generations to FVBN, selecting for the H2b locus in every generation by flow cytometry analyses of H2Kb and H2Db. Afterwards, mice were intercrossed to generate homozygous H2b mice, expressing H2Kb and H2Db, but not H2Kq and H2D/Lq. The haplotype of the strain was tested every 6 to 8 months by flow cytometry (n > 100). This genetic configuration allows K14HPV16/H2b mice to present E7-derived peptides on MHC-I molecules while maintaining the FVBN background that is permissive for squamous carcinogenesis. All the mice were bred in-house and kept under pathogen-free conditions at the animal facility of Ecole Polytechnique Fédérale de Lausanne (Lausanne, Switzerland). Females were subcutaneously implanted with estrogen-releasing pellets at age 1, 3, and 5 months. Five months old females or three months old males were used for the experiments. The presence for cervical carcinomas were confirmed in more than 100 mice by IHC. The TC-1 and SC-1 experiments were performed as described previously (26). The TC-1 cell line was obtained by TC Wu (Johns Hopkins University, Baltimore, MD) in 2014, and SC1 cells were generated in house in 2015 from a squamous cell carcinoma of the skin of a K14HPV16/H-2b mouse. Both cell lines were authenticated by the expression of T-cell peptide SIINFEKL used for restimulation were provided by the Protein and Peptide Chemistry Facility, UNIL and used for NP conjugation and immunization. HPV16 E7 long peptide (aa 43–77, purity>90%) was purchased from Think Peptides and the Protein and Peptide Chemistry Facility, UNIL and used for NP conjugation, immunization, and restimulation of CD8+ T cells. The HPV16 E7 CD8+ T-cell peptide RAYHYNVTF was purchased from Think Peptides and used for restimulation. OVA used for immunization and the OVA CD8+ T-cell peptide SIINFEKL used for restimulation were provided by M. Swartz and J. Hubbell labs. The LCMV CD8+ T-cell peptide KAVYNFATC used for both immunization and restimulation was purchased from the University of Lausanne (UNIL). Ultrapure LPS, used for BMDC activation, was purchased by InvivoGen.

*Immunization*

Mice were immunized as stated in the text with a total amount 15 μg of E7LP or 40 μg of full-length E7 protein, either unconjugated (“free”) or in the NP-bound form and 40 μg of CpG was used as adjuvant. The NP formulations were prepared as indicated below. For the unconjugated formulation, the E7 protein or E7LP were first dissolved in DMSO and then diluted in PBS prior to immunization. Nonimmunized mice were treated with PBS. All the mice from one experiment were immunized together on the same day. Mice received either 1 or 2 shots of vaccine as indicated in the text. Subcutaneous (s.c.) immunizations of mice were performed in the four limbs using the Hock method (immunization volume is 30 μL per location). Intravenous immunizations were performed in the tail vein.

*Nanoparticle synthesis and conjugation*

Nanoparticles (NP) were synthesized, functionalized, and characterized as described previously (26). For antigen conjugation, the HPV16 E7 long peptide (1 mg in 20 μL DMSO) or the full-length E7 protein (1.5 mg in 15 μL DMSO) and incubated for 12 hours in endotoxin-free water in the presence of NPs (500 μL) and guanidine hydrochloride (300 mg, AppliChem) at room temperature. NP-E7LP was purified by size-exclusion chromatography using CL-6B matrix (cat. CL6B200, Sigma-Aldrich) following the manufacturer’s instructions, eluted, and stored in PBS at room temperature. The size of NPs before and after conjugation was determined by dynamic light scattering in a Zetasizer nano-ZS with a modified zeta potential measurement cells (Malvern Instruments Ltd) and remained around 30 nm. E7LP loading on the NPs was measured by BCA assay (Thermo Fisher Scientific). NPs alone have been shown to have no adjuvant activity (27).

*Measurements of cervical tumor size*

Serial 10-μm sections of the optimal cutting temperature (OCT)-embedded cervixes, as described below, were taken and stained with H&E. An image of the whole tissue was taken every 100 μm using an Olympus Slide Scanner VSL120-L100 and processed using Fiji (ImageJ). Tumor area on each of the imaged sections was quantified manually. Tumor volume (V) was calculated using the following formula $V = \frac{1}{2} \times A \times Z$, where A is the maximal tumor area measured on a section and Z is the depth of tumor determined by the number of sections containing tumors.

**Reagents**

CpG-B 1826 oligonucleotide (5’-TTCATGACCTTCTGACGT-3’ as phosphorothioated DNA bases) was purchased from Microsynth and used as adjuvant in various vaccine formulations or to activate BMDCs. The full-length E7 protein was purchased from the Protein and Peptide Chemistry Facility, UNIL and used for NP conjugation and immunization.
**Table 1. Treatments.**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dosage</th>
<th>Frequency</th>
<th>Route</th>
<th>Volume</th>
<th>Source</th>
</tr>
</thead>
<tbody>
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<td>Anti-PD-1 (Fig. 2)</td>
<td>200 μg/mouse</td>
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<td>200 μL</td>
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<td>IP</td>
<td>200 μL</td>
<td>Bristol-Myers Squibb</td>
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<tr>
<td>Anti-CTLA4 (mCTLA4-9D9-mlgG2c, clone 2B7_RAS_Ab, O2, Fig. 7 and Supplementary Fig. S28)</td>
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<td>2x week</td>
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<td>Bristol-Myers Squibb</td>
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<tr>
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<td>Sigma</td>
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<tr>
<td>Paclitaxel</td>
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<td>200 μL</td>
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<td>IP</td>
<td>200 μL</td>
<td>Bioxcell, clone RB6-8C5</td>
</tr>
</tbody>
</table>

**Abbreviation:** IP, intraperitoneal.

replaced media were recovered and put back in culture). At day 8, the BM DCs in suspension were collected and used for the different assays.

**DC vaccine (DC-VAX) preparation**

BMDCs were collected at day 8 of culture. Cells were incubated with E7LP for 3 hours at 37°C for antigen loading. E7LP-loaded BMDCs were activated with 20 ng/mL LPS for 12 hours. After the activation, the cells were extensively washed to remove the excess of LPS, harvested, and mixed with CpG. For certain experiments, when stated in the text, BMDCs were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE, 2 μM/L) prior to mixing with CpG. Each mouse received a total of 2 × 10^7 BMDCs and 40 μg of CpG either subcutaneously or intravenously as indicated.

**Coculture experiments with BMDCs**

BMDCs were collected at day 8 of culture as previously illustrated. Cells were activated for 12 hours with either LPS (20 ng/mL) or CpG (0.1 μM/L) as indicated in the text. After the activation, the cells were extensively washed with PBS and centrifuge to remove the excess of LPS and CpG and plated together with Tregs, CD4^+^-T-reg cells, or CD11b^+^ cells isolated from K14HPV16/H2b or FVBN/H2b mice as indicated. The ratio of the coculture was 1 BMDC to 10 other cells. BMDCs were labeled with CFSE (2 μM/L) prior to mixing with CD11b^+^ cells. After 24 hours of coculture, cells were harvested and processed for flow cytometry analysis.

**Cell preparation for flow cytometry, antigen-specific in vitro restimulation, and magnetic isolation**

Spleens or lymph nodes were harvested and gently disrupted through a 40-μm filter (Thermo Fisher Scientific) in iscove’s modified Dulbecco medium (IMDM; Gibco). Red blood cells were lysed using ACK lysis buffer (not for magnetic isolation), and cells were filtered again through a 40-μm filter before use. For CD8^+^-T cell–antigen–specific restimulation, cell suspensions from either spleen or lymph nodes were cultured at 37°C for 4 hours in a 96-well plate in the presence of 1 μg/mL of the HPV16 CD8^+^-T cell peptide RAHYNIVTF. After the first 3 hours of culture, brefeldin A (Sigma-Aldrich) was added to a final concentration of 5 μg/mL. All the cells were cultured in IMDM (Gibco) supplemented with 10% FBS (Gibco) and 1 × penicillin/streptomycin (100 U/mL penicillin, 100 μg/mL streptomycin; Gibco).

**Coculture experiments with CD8^+^-T cells**

CD8^+^-T cells were isolated from naïve mice or from mice that were previously immunized against E7 as stated in the text. CD8^+^-T cells isolation was performed on the spleen using the Easysep Mouse CD8^+^-T cell Isolation Kit (catalog no. 19853, StemCell Technologies) for magnetic isolation according to the manufacturer’s instructions. CD8^+^-T cells purity was assessed by flow cytometry and above 90% in all the experiments. For proliferation assays, CD8^+^-T cells were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE, 2 μM/L) and added to a plate that has previously been incubated for 12 hours with 2 μg/mL anti-CD3/anti-CD28 in PBS at 4°C. CD8^+^-T cells were harvested 48 hours later, stained with fixable live/dead cell viability reagent (LIVE/DEAD Fixable Violet Dead Cell Stain Kit, Invitrogen) diluted in PBS for 15 minutes on ice and analyzed by flow cytometry to assess their proliferation. For restimulation experiments, isolated CD8^+^-T cells were added to a plate, and the assay was performed as previously illustrated. When indicated, CD8^+^-T cells were cocultured with other cell types in a 1 CD8^+^-T cell to 10 other cell ratio. Samples were acquired on a Cyan or Gallios analyzer (Beckman Coulter) and data were analyzed using FlowJo software.

**Cell isolations for coculture experiments and neutrophil sorting**

To obtain enough cells to perform the various assays, cell isolations were performed on the spleen. Tregs were isolated using a double step magnetic isolation CD4^+^-CD25^+^-Regulatory T Cell Isolation Kit (catalog no. 130-091-041, Miltenyi Biotec); cell purity was assessed by flow cytometry and around 70% in all the experiments. CD11b^+^-T cells were isolated using the magnetic isolation–positive selection Easysep Mouse CD11b Positive Selection Kit II (catalog no. 18970, StemCell Technologies). Collectively, monocytes, neutrophils, macrophages, and DCs accounted for more than 80% of the isolated cells. Monocytes were isolated using the magnetic isolation–negative selection Easysep Mouse Monocyte Isolation Kit (catalog no. 19861, StemCell Technologies); cell purity was assessed by flow cytometry and higher than 80%. Macrophages were isolated using the magnetic isolation Easysep PE positive selection kit (catalog no. 17684, StemCell Systems).
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**Protein extractions and Western blot analysis**

Pituitary and brachial lymph nodes were harvested from three months old naïve untreated mice. Lymph nodes were snap frozen upon collection. Lymph nodes were put in RIPA buffer with protease (cOmplete Mini, Roche) and phosphatase (PhosSTOP, Roche) inhibitors and mechanically lysed using an Eppendorf microcurette. CD11b+ cells were isolated from the spleen as previously described and the CD11b+ splenocytes were also recovered from the kit. Cells were resuspended in RIPA buffer. Samples were centrifuged at 10,000 × g, and the supernatant was collected and used for Western blot analysis. Protein concentration was determined by Bradford assay (Advanced Analytical). The same amount of protein was used for the Western blots. The same amount of total protein was loaded in each well. The following antibodies were used: β-actin (catalog no. 48675, Cell Signaling Technology), G-CSF (clone EPR3203(N)), GM-CSF (clone MP122E9, R&D Systems), M-CSF (catalog no. AB99178, Abcam), IL-10 (clone JES552A5, R&D Systems), IDO (clone mIDO-48, BioLegend), IL-1β (catalog no. AB9722, Abcam), HSP90 (clone F-8, Santa Cruz Biotechnology), and C-reactive protein (CRP, catalog no. AF1829, R&D Systems). Peroxidase-conjugated anti-rat antibody was from Cell Signaling Technology. Peroxidase-conjugated anti-rabbit, anti-goat, and anti-mouse were from Dako. Secondary antibodies were used at 1:3,000 dilution. Proteins were detected by enhanced chemiluminescence (ECL) Westernbright sirius (Advansta). Western blot bands were visualized on a Fusion FX and quantified using Fiji (ImageJ).

**RNAseq on neutrophils**

Live CD11b+Ly6G+Ly6C- neutrophils were FACs sorted, and total RNA was isolated with the mirNeasy Micro Kit (Qiagen). The quality and quantity of the RNA was determined by a Fragment Analyzer (Advanced Analytical). The RQN for the RNA ranged from 9.6 to 10. Library preparation and sequencing was performed at the Lausanne Genomic Technologies Facility (University of Lausanne, Lausanne, Switzerland). Double-stranded cDNA for RNAseq library preparation was generated using SMART-Seq v4 Ultra Low Input RNA reagents (catalog no. 634888, ClonTech) according to the protocol provided in the relevant gating strategy was applied. Antibodies used for sorting: CD11b (clone M1/70, Thermo Fisher Scientific), Ly6G (clone 1A8, BioLegend), Ly6C (clone HK1.4, Thermo Fisher Scientific). The gating strategy is shown in the Supplementary Fig. S1.

**Reactive oxygen species production assay**

CD11b+ cells were isolated as previously described and cultured in a 96-well plate for 1 hour at 37°C in complete RPMI. Media was then removed, and the reactive oxygen species (ROS)–reactive dye 2′,7′-Dichlorofluorescin diacetate (DCFDA, Sigma) was added at a final concentration of 10 μg/mL in PBS, and the cells were left for 30 minutes at 37°C. Phorbol 12-myristate 13-acetate (PMA) at a final concentration of 1 μg/mL was used as positive control for ROS production. At the end of the experiment, cells were harvested, labeled with fixable live/dead cell viability reagent (LI/DEAD Fixable Violet Dead Cell Stain Kit, Invitrogen) diluted in PBS for 15 minutes on ice and analyzed by flow cytometry. Samples were acquired on a Cyan or Gallios analyzer (Beckman Coulter) and data were analyzed using FlowJo software (Tree Star Inc.).

**Flow cytometry and gating strategy**

For blocking and surface staining, cells were incubated for 15 minutes on ice with the antibodies diluted in PBS 2% FBS. Before tetramer and antibody staining, all cell suspensions from tumor or spleen were blocked with anti-CD16/32 (Clone 93, BioLegend). Cells were then labeled with fixable live/dead cell viability reagent (LI/DEAD Fixable Red Dead Cell Stain Kit, Invitrogen) diluted in PBS for 15 minutes on ice. Staining with a tetramer recognizing HPV16 E7 peptide 49–57 presented by H2Db (University of Lausanne, Lausanne, Switzerland) was performed before antibody staining for 30 minutes at room temperature. Tetramer staining for OVA-specific and LCMV-specific CD8+ T cells was performed similarly using tetramers recognizing the SIINFEKL (Proimmune) and KAVYNFATC (University of Lausanne, Lausanne, Switzerland) peptide–MHC complexes. If no intracellular staining was performed after surface staining, cells were fixed with 2% PFA in PBS for 15 minutes on ice. For intracellular staining, cells were permeabilized and fixed with the Fixop3/Transcription Factor Staining Buffer Set Kit (eBioscience) following the manufacturer instructions and then incubated overnight at 4°C with the antibodies diluted in 1× Permeabilization buffer provided with the aforementioned kit. After staining, cells were washed and resuspended in PBS 2% FBS for analyses. Samples were acquired on a Cyan or Gallios analyzer (Beckman Coulter) and data were analyzed using FlowJo software (Tree Star Inc.). The gating strategies are shown in Supplementary Figs. S2–S8, including the information in which figures the relevant gating strategy was applied. Antibodies used for flow cytometry: CD3 (clone 145–2C11, Thermo Fisher Scientific), CD4 (clone RM4-5, BioLegend), CD8a (clone 5H10, Thermo Fisher Scientific), B220 (clone RA3–82R, Thermo Fisher Scientific), IFNγ (clone XMG1.2, BioLegend), TNFα (clone MP6-XT22, Thermo Fisher Scientific), Foxp3 (clone FJK-16s, Thermo Fisher Scientific), CD25 (clone PC61.5, Thermo Fisher Scientific), CD11b (clone M1/70, Thermo Fisher Scientific), CD11c (clone N418, BioLegend), Ly6G (clone 1A8, BioLegend), Ly6C (clone HK1.4, Thermo Fisher Scientific), MHCII

**Gene expression analysis.** Protein concentration was determined by Bradford assay (Tree Star Inc.). Staining was performed as described in the flow cytometry section. Antibodies used for sorting: CD11b (clone M1/70, Thermo Fisher Scientific), Ly6G (clone 1A8, BioLegend), Ly6C (clone HK1.4, Thermo Fisher Scientific). The gating strategy is shown in the Supplementary Fig. S1.

**Gene set enrichment analysis (28)** in the Hallmark and KEGG collections of MSigDB v5.0 with 100,000 permutations andBonferroni corrected FDR adjusted P < 0.05 were considered significant. All statistical analyses were performed using the free high-level interpreted statistical language R (version 3.5.1) and various Bioconductor packages (http://bioconductor.org). Data are publicly available in GEO database (GSE126913).

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Cervixes were harvested, embedded in OCT (Sakura), and snap frozen on dry ice. Ten-μm-thick sections were cut from OCT-embedded samples using a cryostat and collected on Superfrost Plus glass slides (Thermo Fisher Scientific). Tissue sections and OCT-embedded samples were stored at -80 °C. For immunofluorescence staining, sections were fixed in ice-cold methanol (Thermo Fisher Scientific) for 10 minutes before proceeding. Slides were washed with PBS to remove the remaining OCT and then blocked for 45 minutes at room temperature with PBS + 5% BSA + 2.5% FBS and then stained with primary antibodies diluted in PBS + 1% BSA overnight at 4 °C in a humidified chamber. On the following day, slides were washed with PBS and stained with secondary antibodies diluted in PBS + 1% BSA for 1 hour at room temperature. Before mounting, slides were washed again in PBS and then covered with mounting media (Dako) containing DAPI (Roche, 5 μg/mL). Coverslips (Menzel Glaser) were applied to the slides and sealed using nail polish. Images were acquired using a Leica DM5500B or an Olympus Slide Scanner VSI.120-L100 and processed using Fiji (ImageJ). Antibodies used for immunofluorescence staining: CD8 (clone 53–6.7, Bioscience), keratin 14 (clone poly19053, Biolegend), CD3 (clone 500A2, BD), and PD-L1 (clone MIH5, eBioscience).

TCRβ deep sequencing of E7-specific CD8 T cells

Lymph nodes were processed as described above to obtain a single-cell suspension. Cells were stained with APC tetramers recognizing HPV16 E7 peptide 49–57 presented by H2Db (University of Lausanne, Lausanne, Switzerland) and E7-specific CD8 T cells were isolated using the EasySep APC Positive Selection Kit II (catalog no. 18452, StemCell Technologies) following the manufacturer’s instructions. Total genomic DNA was extracted using the QIAamp DNA Micro Kit (Qiagen). TCRβ sequencing was performed using the two-stage PCR method as described previously (29). Briefly, the first stage involved amplifying the gDNA and the synthetic TCR template (internal controls) using a multiplex PCR containing a E7-synthetic long peptide (E7LP) conjugated to NPs for the therapeutic vaccine (26). Using a 2% 0.5 μM of purified PCR product from stage 1 as template, we performed a second stage PCR with universal and indexed Illumina adapters. TCRβ library was prepared by pooling equal volumes of the second stage PCR products. The library was profiled on a 2200 TapeStation (Agilent) and the concentration was determined by real-time PCR using a StepOne cycler (Applied Biosystems). The library was processed using the MiXCR tool suite. Normalized clonotype counts were exported in tabular format for use in downstream analysis. TCR repertoire metrics, including clonality, maximum clonal frequency, and the Shannon diversity index were calculated using the methods described in Medler and colleagues (29).

Statistical analyses

Statistical analyses were performed in GraphPad Prism 7. Flow cytometry, tumor size and Western blot quantification data were compared using two-tailed Student t test or one-way ANOVA, as indicated in the figure legends. TCRβ deep sequencing results were compared using Mann–Whitney test. Statistical significance is indicated as follows: *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001; n.s., not significant. All error bars are ± SEM or ± SD, as indicated in the figure legends.

Results

The K14HPV16/H2b mouse model

Here we employed the K14HPV16/H2b mouse model to study the therapeutic efficacy of E7-directed immunotherapies. Similar to the original K14HPV16 mouse (24, 25, 30, 31), this refined model expressed the early region of HPV16 under the human keratin 14 promoter (Fig. 1A) and, upon continuous estrogen treatment (Fig. 1B), female mice developed de novo cervical carcinomas (Fig. 1C) via progressive cervical intraepithelial neoplasias that phenocopy the multistage progression implicated in human cervical carcinogenesis (31). Because of the widespread expression of HPV16 genes in basal keratinocytes of the skin, the prototypical K14HPV16 mice also develop skin lesions characterized by hyperkeratosis and dysplasia, and squamous cell carcinomas later in life (24, 25, 32), phenotypes that are also observed in K14HPV16/H2b mice. This new mouse line expresses the H2Db and H2Kb class I MHC molecules, as a result of introducing the H2b locus from the C57Bl/6 background (Fig. 1D), required for E7 peptide presentation. To assess the capability of this congenic line to mount a CD8+ T-cell response toward HPV16 neoantigens, we immunized the mice against E7. As reported previously, antigen conjugation to NPs can boost the therapeutic efficacy of an E7-therapeutic vaccine compared with a classical unconjugated formulation (26). Five-month-old K14HPV16/H2b female mice received the vaccine containing the full-length E7 protein at day 0 and 14 and were sacrificed at day 19 to assess the immune response in lymph nodes. The unconjugated (“liquid”) formulation was unable to elicit a measurable response in transgenic mice, whereas the NP-E7 vaccine produced detectable albeit modest numbers of E7-specific CD8 T cells (Supplementary Fig. S9). No spontaneous response was evident in unvaccinated mice (Supplementary Fig. S9). Thus, E7-specific CD8+ T cells can be generated in K14HPV16/H2b mice.

Immunotherapy was ineffective in K14HPV16/H2b mice

We next assessed the therapeutic efficacy of the NP vaccine in combination with ICB in K14HPV16/H2b mice (Fig. 2). In these and subsequent experiments, we switched to a vaccine formulation containing a E7-synthetic long peptide (E7LP) conjugated to NPs (NP-E7LP) (26), to model similar formulations with this peptide used in clinical trials (13–17).

Five-month-old female mice with cervical carcinomas were immunized with the therapeutic vaccine alone or in combination with anti-PD-1 and anti-CTLA4, given that PD-L1 expression was detected on cervical tumors (Supplementary Fig. S10). Antibody treatment started on day 0 of the vaccination. Flow cytometry analysis of the immune response performed 24 days postvaccination revealed that ICB was unable to increase the abundance of E7-specific CD8+ T cells (Fig. 2A). Upon ex vivo restimulation, cytokine production was also similar between the two treatment groups (Fig. 2B). In addition, neither treatment had an impact on the tumor size (Fig. 2C) and we observed
no obvious changes in CD3⁺ or CD8⁺ T-cell infiltrates in the TME of the cervix (Fig. 2D, representative field; Supplementary Fig. S11, entire cervical tumor). These results contrast previously observed data in mice bearing transplanted SC-1 and TC-1 tumors, in which CD8⁺ T-cell infiltrates were robustly increased upon vaccination with NP-E7LP (Fig. 2E; ref. 26). This dichotomy implicated immunosuppressive barriers in K14HPV16/H2b mice capable of blocking the effects of the oncoprotein vaccine, alone and in combination with ICB. Similarly to what has been observed in patients with cervical cancer (14, 17, 33), these data reveal that immunotherapy is poorly effective against de novo formed tumors present in K14HPV16/H2b mice.

The immune response to vaccination was weaker in K14HPV16/H2b compared with FVBN/H2b mice

Given the widespread expression of the HPV oncoproteins in K14HPV16/H2b transgenic mice, we hypothesized that these mice might exhibit partial tolerance toward E7, leading to the generation of T cells with reduced effector function and impaired migration to tumors. To investigate this possibility, we immunized 5-month-old females and 3-month-old males and compared K14HPV16/H2b mice with their WT FVBN/H2b littermates (Fig. 3). Mice were vaccinated once, and the immune response was measured 9 days later in the lymph nodes draining the vaccination site (Fig. 3). Flow cytometry analysis showed similar immune responses regardless of the sex and

Figure 1.
Systemic Myeloid Immunosuppression in HPV+ Cervical Cancer

Immunotherapy had no antitumor efficacy against cervical cancer in K14HPV16/H2b mice. A, E7-specific CD8+ T cells in the spleen (PBS, n = 6; VAX, n = 4; VAX aPD-1 aCTLA4, n = 5). B, IFNγ and TNFα production from CD8+ T cells (PBS, n = 6; VAX, n = 4; VAX aPD-1 aCTLA4, n = 5). C, Cervical tumor size (PBS, n = 5; VAX, n = 9; VAX aPD-1 aCTLA4, n = 5). D, Immunofluorescent staining for CD8 (green), CD3 (red), keratin 14 (K14, white), and nuclei (blue) of cervical tumors. E, Immunofluorescent staining for CD8 (green), keratin 14 (K14, white, SC-1 only), and nuclei (blue) on SC-1 and TC-1 tumors (PBS, n = 4; VAX, n = 4). Scale bars, 100 μm. Independent experiments: two (A–D), five (E). All panels, t-test, error bars ± SEM (*, P < 0.05; **, P < 0.01; ††, P < 0.001; †††, P < 0.0001; ns, not significant).

Figure 2.

of the mice, with K14HPV16/H2b mice exhibiting fewer E7-specific CD8+ T cells compared with FVBN/H2b controls (Fig. 3A–D). These results indicated that an impaired immune response was characteristic of the HPV transgenic mice and was not solely caused by the cervical tumors present only in female mice. This impairment might have been due to the widespread expression of E7 in...
K14HPV16/H2b mice had a weaker systemic immune response to therapeutic vaccination compared with FVBN/H2b mice. E7-specific CD8+ T cells in the lymph nodes after immunization of 5-month-old females (A) or 3-month-old males (B). Tetramer staining for E7-specific CD8+ T cells in females (C) or males (D). E, E7-specific CD8+ T-cell phenotype analyses in females. F, IFNγ and TNFα production from CD8+ T cells. G, IFNγ and TNFα production from CD4+ T cells. H, Clonal index measured from TCRβ deep sequencing of E7-specific CD8+ T cells. Cumulative frequencies of hyperexpanded (>0.01; I) and large (0.001–0.01; J) clones. K, CD8+ T-cell proliferation. L, IFNγ and TNFα production of CD8+ T cells isolated from the spleen. M, OVA-specific CD8+ T cells in lymph nodes. N, IFNγ and TNFα production from CD8+ T cells. O, LCMV-specific CD8+ T cells in lymph nodes. P, IFNγ and TNFα production from CD8+ T cells. Females, n = 6 (A); males, n = 4 (B, E–G); E7-specific CD8+ TCR sequencing, n = 8 (H–J); CD8+ T-cell proliferation and cytokine production, n = 5 (K and L); OVA and LCMV immunization, n = 5 (M–P). Independent experiments: three (A–E), one (F), two (G), one (H–P). All panels: t test, except E and F: ANOVA, error bars ± SEM (*, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001; ns, not significant).
K14+ keratinocytes of the skin, where hyperplasias and dysplasias but not overt squamous carcinomas arise in mice at these ages (25).

We further characterized the impaired immune response, finding that the effector/memory phenotypes of E7-specific CD8+ T cells were altered, with fewer CD62L+CD44+ effector and CD44+KLRG1+ terminal effector cells, and a relatively higher percentage of CD62L+CD44+ memory cells in K14HPV16/H2b mice compared with nontransgenic FVBN/H2b controls (Fig. 3E). These differences suggested that antigen-specific CD8+ T cells undergo limited expansion and differentiation into effector cells upon encounter with their cognate antigen in the transgenic mice. After ex vivo restimulation, cytokine production from E7-specific T cells was lower in K14HPV16/H2b mice for both CD8+ and CD44+ T cells as compared with T cells from FVBN/H2b mice (Fig. 3F and G; Supplementary Fig. S12A and S12B). Cytokine production from CD8+ T cells in K14HPV16/H2b mice did not increase, even upon increased concentration of the cognate peptide (Fig. 3F).

Deep sequencing of the beta chain of the T-cell receptor (TCR) from E7-specific CD8+ T cells revealed a lower clonality (Fig. 3H) in K14HPV16/H2b mice as compared with FVBN/H2b mice, which was associated with fewer hyperexpanded and large frequency clones (Fig. 3I and J; Supplementary Fig. 13A). The clonal frequency of the most highly expanded clonotype was also significantly lower in the transgenic mice (Supplementary Fig. S13B), while no differences were apparent in Shannon diversity index (Supplementary Fig. S13C) or in number of unique clones (Supplementary Fig. S13D). Although some hyperexpanded clones present in FVBN/H2b mice were also identified in the K14HPV16 mice, their frequencies were generally lower in transgenic mice (Supplementary Fig. S13E), indicating that E7-specific T-cell clones were evidently unable to expand to sufficient numbers in transgenic mice to be detected.

To exclude the possibility that this impaired T-cell phenotype was a cell-intrinsic defect, we isolated CD8+ T cells from K14HPV16/H2b and FVBN/H2b mice and performed in vitro proliferation and cytokine production assays. CD8+ T-cell proliferation, measured by CFSE dilution upon anti-CD3/anti-CD28 activation, was similar between the two strains (Fig. 3K). IFNγ and TNFα production from anti-CD3/anti-CD28–activated CD8+ T cells was higher (3-fold) in the cells isolated from K14HPV16/H2b mice (Fig. 3L). These data indicated that CD8+ T cells from transgenic K14HPV16/H2b mice have no cell-intrinsic defects in their proliferation and function, suggesting that their impaired expansion and activity was the result of external factors.

Immunosuppression in K14HPV16/H2b mice abrogated immune responses to foreign antigens

If the above analyses were consistent with a condition of partial immune tolerance, then the impairment would be specific to the E7 neo-self-antigen, whereas the immune response to non-self-antigens should be similar in transgenic and nontransgenic mice. To address this possibility, we immunized 3-month-old K14HPV16/H2b or FVBN/H2b male mice with two non-self-antigens: chicken ovalbumin (OVA) and an LCMV-derived peptide recognized by CD8+ T cells. K14HPV16/H2b mice exhibited lower OVA-specific CD8+ T cells (Fig. 3M) and lower cytokine production upon ex vivo restimulation with the OVA-derived CD8+ T cell–specific peptide SIINFEKL (Fig. 3N) compared with their WT FVBN/H2b littermates. As for the immunization with the LCMV-derived peptide, although the abundance of LCMV-specific CD8+ T cells was similar between the
two strains (Fig. 3O), cytokine production after ex vivo restimulation was severely impaired in K14HPV16/H2b mice (Fig. 3P).

Thus, although we could not completely exclude the existence of partial self-tolerance to the E7 protein, it was evident that the impaired immune response seen in K14HPV16/H2b mice was not restricted to the E7 antigen. Hence, these results suggested that a systemic immunosuppression mechanism was occurring.

**Activation of APCs was suppressed in K14HPV16/H2b mice**

Next, we sought to determine whether antigen presentation by APCs (in particular DCs) was directly affected by the immunosuppressive mechanism evident in K14HPV16/H2b mice. To do so, we subcutaneously immunized K14HPV16/H2b mice and their FVBN/H2b littermates with the NP-E7LP vaccine and assessed the activation of DCs harvested from the lymph nodes draining the vaccination site after 24 hours. CD11b<sup>−</sup>CD11c<sup>+</sup> DCs from FVBN/H2b mice showed a significant upregulation of all the analyzed markers, whereas DCs from K14HPV16/H2b mice only upregulated CD86 (Fig. 4A), albeit to a significantly lower extent compared with the FVBN/H2b counterpart. No upregulation of CD80, CD40, and MHCII was detected in K14HPV16/H2b mice (Fig. 4A), indicating that DC activation was markedly impaired in HPV transgenic mice. Similar to patients with cervical cancer (21), K14HPV16/H2b mice had decreased numbers of DCs in the spleen, as measured by flow cytometric analysis (Supplementary Fig. S14A); in contrast, there was no difference in the lymph node populations (Supplementary Fig. S14B), suggesting that the weaker immune response measured in the GEMM cannot be explained by insufficient DC abundance there.

We next isolated DCs from the bone marrow of K14HPV16/H2B mice or from their FVBN/H2b littermates, aiming to assess their functionality. In vitro activation experiments with either LPS or CpG revealed that upregulation of activation markers was similar between BMDCs from either strain (Supplementary Fig. S14C), indicating that the impaired DC activation was not cell intrinsic and was not related to the use of CpG, whose receptor, TLR9, can be downregulated by HPV16 (34).

Given that the endogenous DCs were functionally impaired, we reasoned that it might be possible to rescue the immune response by administrating antigen-loaded, activated DCs as an anti-E7 DC-vaccine (Supplementary Fig. S14D). To avoid any possible interference from the HPV16 transgenes, we generated BMDCs from the BM of a syngeneic FVBN/H2b mouse. Activated BMDCs were loaded with E7LP, and injected into K14HPV16/H2b or FVBN/H2b mice. The administration was performed either subcutaneously or intravenously to trigger the generation of an anti-E7 response in lymph nodes or spleen, respectively. Additional groups of mice received CFSE-labeled BMDCs and were sacrificed 24 hours after the administration to assess their activation (Supplementary Fig. S14D). Regardless of the administration route, the expression of CD86, CD40 and MHCII showed a trend pointing to an impaired activation status of the transferred BMDCs in K14HPV16/H2b mice (Supplementary Fig. S14E and S14F), suggesting that they were being actively suppressed. When mice that received the unlabelled BMDC vaccine were sacrificed 9 days after immunization, we found similar data to what was described above for NP-E7LP vaccination. Namely, there were fewer E7-specific CD8<sup>+</sup> T cells (Fig. 4B; Supplementary Fig. S14G), with an altered phenotype in both lymph nodes and spleen (Fig. 4C; Supplementary Fig. S14H, respectively) and lower cytokine production by CD8<sup>+</sup> T cells after restimulation (Fig. 4D). These data indicated that adoptively transferred DCs were being actively suppressed in both lymph nodes and spleen of K14HPV16/H2b mice, like endogenous DCs.

**Myeloid cells were orchestrating systemic immunosuppression in K14HPV16/H2b mice**

Our data collectively indicated that the T-cell responsiveness in K14-HPV16/H2b mice was being suppressed (in paracrine fashion) in lymphoid organs. We reasoned that an immunosuppressive immune cell population could be mediating the observed effects and sought to investigate the possibility.

Previous reports have revealed that Tregs might play a role in HPV E7-mediated immunosuppression (23, 35). Flow cytometry analysis of K14HPV16/H2b mice and their FVBN/H2b littermates showed that the number of Tregs was similar in spleen and slightly higher in lymph nodes of K14HPV16/H2b mice (Supplementary Fig. S15A and S15B). However, their phenotype was substantially different: CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Treg cells expressing the IL-7 receptor CD127 were increased in K14HPV16/H2b mice (Supplementary Fig. S15C and S15D). Notably, CD127<sup>+</sup> Tregs are reportedly more efficient at sequestering IL-2, a cytokine required for optimal T-cell expansion during immune responses (36).

To test the suppressive activity of Tregs, we assessed the impact of Tregs on CD8<sup>+</sup> T-cell proliferation and cytokine production, and APC activation by in vitro coculture assays (Supplementary Fig. S15E). As expected, CD8<sup>+</sup> T-cell proliferation was suppressed by Tregs compared with conventional CD4<sup>+</sup> T cells; however, we detected no difference in suppression between Treg s derived from K14HPV16/H2b versus FVBN/H2b mice (Supplementary Fig. S15F). To ascertain the effects of Tregs on cytokine production from E7-specific CD8<sup>+</sup> T cells, we immunized an FVBN/H2b mouse with the NP-E7LP vaccine and isolated CD8<sup>+</sup> T cells 9 days later (Supplementary Fig. S15G). Similar to the previous experiments, Treg s from the K14HPV16/H2b-derived populations were then restimulated in the presence of Treg s or conventional CD4<sup>+</sup> T cells isolated from either K14HPV16/H2b or FVB/H2b mice (Supplementary Fig. S15H). Once again, Tregs from both strains suppressed cytokine production in a similar manner compared with conventional CD4<sup>+</sup> T cells (Supplementary Fig. S15I).

To assess the effects of Tregs on APCs, we generated BMDCs from an FVBN/H2b mouse, loaded them with E7LP, and activated them with LPS. On the following day, BMDCs were mixed with Treg s or with conventional CD4<sup>+</sup> T cells, and cocultured for 24 hours (Supplementary Fig. S15J). The expression of activation markers was similar between BMDCs cultured with K14HPV16/H2b or FVBN/H2b-derived cells, both for Treg s or conventional CD4<sup>+</sup> T cells (Supplementary Fig. S15K), with no sign of increased suppression from the K14HPV16/H2b-derived populations. Collectively, these results indicated that Tregs had similar activity in both strains. These data suggest that Tregs were not responsible for the selectively impaired immune responses seen in K14HPV16/H2b transgenic mice.

We next focused on myeloid-derived suppressor cells (MDSC) that have immunosuppressive and tumor-promoting capabilities, although most studies have focused on their presence and functions within the TME (37). Consistent with the enlarged spleen and lymph nodes (Supplementary Fig. S16), all major myeloid cell populations were significantly more abundant in these organs of K14HPV16/H2b mice (Fig. 5A-D). To determine whether myeloid cells from K14HPV16/H2b mice possessed immunosuppressive capabilities, we performed an in vitro coculture experiment with CD11b<sup>+</sup> myeloid cells, similar to the coculture experiment performed with the Tregs. Both CD8<sup>+</sup> T-cell proliferation (Fig. 5E; Supplementary Fig. S17A and S17B) and cytokine production (Fig. 5F; Supplementary Fig. S17C) were significantly lower when FVBN/H2b-derived CD8<sup>+</sup> T cells (from untreated mice and after NP-E7LP immunization, respectively) were cocultured with CD11b<sup>+</sup> myeloid cells isolated from the spleens of K14HPV16/H2b...
Systemic Myeloid Immunosuppression in HPV+ Cervical Cancer

Myeloid cells were expanded in lymphoid organs and possessed suppressive activity toward CD8+ T cells and DCs in K14HPV16/H2b mice. Flow cytometry analysis on lymph nodes (A) and spleen (B) of total CD11b+ myeloid cells, CD11b+Ly6G+Ly6C- G-MDSCs/neutrophils, CD11b+Ly6G+Ly6C+ Mo-MDSCs/monocytes, and CD11b+Ly6G+Ly6C+ F4/80+ macrophages (3-month-old males, n = 6). G-MDSCs, granulocytic MDSCs; Mo-MDSCs, monocytic MDSCs. Plots of CD11b+ cells in lymph nodes (C) and spleen (D). E, CD8+ T-cell proliferation in the presence of CD11b+ cells or myeloid cell-depleted CD11b+ cells isolated from the indicated genotype (n = 4). F, IFNγ and TNFα production from CD8+ T cells in the presence of CD11b+ myeloid cells from the spleen of K14HPV16/H2b or FVBN/H2b mice (n = 5). G, CD86, CD40, and MHCII expression on BMDCs after coculture with CD11b+ cells from the spleen of K14HPV16/H2b or FVBN/H2b mice (nonactivated BMDCs, BMDCs only, FVBN/H2b/CD11b+ cells, n = 4; K14HPV16/H2b CD11b+ cells, n = 6). MFI, mean fluorescence intensity. H, CD8+ T-cell proliferation in the presence of monocytes from the spleen of K14HPV16/H2b or FVBN/H2b mice (CD11b+ monocytes, n = 5; K14HPV16/H2b monocytes, n = 6). I, CD8+ T-cell proliferation in the presence of macrophages from the spleen of K14HPV16/H2b or FVBN/H2b mice (n = 6). Independent experiments: three (A-D), three (E and F), two (G), one (H and I). All panels: t test, error bars = SEM (**, P < 0.01; ***, P < 0.001; ****, P < 0.0001; ns, not significant).

H2b mice. When CFSE-labeled CD8+ T cells were cocultured in the presence of myeloid cell-depleted CD11b+ splenocytes (the cell fraction recovered after CD11b+ cell isolation), there was no inhibition of their proliferation (Fig. 5E; Supplementary Fig. S17B). These results indicated that active immunosuppression was conveyed by CD11b+ myeloid cells. BMDC activation (Supplementary Fig. S17D) was also lower in cells that were cultured in the presence of CD11b+ myeloid cells derived from K14HPV16/H2b mice compared with the coculture with FVBN/H2b-derived CD11b+ cells (Fig. 5G). Collectively, these data demonstrated that myeloid cells derived from lymphoid organs of...
K14HPV16/H2b mice had the capability to impair the functions of both CD8^+ T cells and DC/APCs, and were likely responsible for the systemic suppression of immune responses observed in the HPV transgenic mice.

We next sought to determine which of the major cell subpopulations contained within the CD11b^+ umbrella possessed suppressive activity. We focused on monocytes, macrophages, and neutrophils. As above, we isolated the different cell populations and performed the coculture assay scoring for effects on CD8^+ T-cell proliferation. Monocytes (Fig. 5H) but not macrophages (Fig. 5I) showed suppressive activity. Because we could not perform in vitro assays with neutrophils due to their very brief survival after isolation (38), we performed RNaseq to compare K14HPV16/H2b-derived with FVBN/H2b-derived neutrophils. Sorted CD11b^+Ly6G^+Ly6C^- neutrophils from K14HPV16/H2b or FVBN/H2b mice clustered separately, with 761 genes significantly differentially expressed, indicating that neutrophil transcriptome profiles are substantially different between the two strains (Supplementary Fig. S18). Pathway analyses using the KEGG and Hallmark genesets (Supplementary Fig. S19) revealed an upregulation of several inflammatory and metabolic pathways in neutrophils from K14HPV16/H2b transgenic mice including the IL-6–JAK–STAT3 signaling pathway (Supplementary Fig. S20). Immune cell signaling via STAT3 is proposed to have immunosuppressive activity in patients with cervical cancer (22), and both IL-6 and STAT3 are linked with the suppressive functions of MDSCs (37, 39), implicating neutrophils and monocytes in the systemic immunosuppression evident in K14HPV16/H2b mice.

**Increased immunosuppressive and myeloid cell regulatory factors in K14HPV16 mice**

We then analyzed the protein expression of candidate immunosuppressive factors in the draining lymph nodes (dLN) of the vaccination site, looking for additional local factors that could be involved in the underlying suppressive mechanism, in addition to the systemic factors suspected to mediate the expansion/alteration of the myeloid cell compartment. IL–10 and IDO were both found increased in the dLNs and CD11b^+ cells of K14HPV16/H2b mice compared with FVBN/H2b mice confirming that these factors were produced by K14HPV16/H2b myeloid cells (Fig. 6B; Supplementary Figs. S21 and S22). In addition, we observed a significant increase in ROS production by CD11b^+ cells in K14HPV16/H2b mice compared with FVBN/H2b (Fig. 6C). Thus, myeloid cells from K14HPV16/H2b mice expressed immunosuppressive factors and were more abundant, implicating them in the immune suppression.

Concurrently, we analyzed the lymph nodes for factors known to be involved in the regulation of myeloid cell expansion. Notably, we observed a significant increase in GM–CSF and G–CSF but not M–CSF in K14HPV16/H2b mice (Fig. 6A; Supplementary Fig. S21). We also observed a general increase in the inflammation-related factors C-reactive protein, IL–1β and HSP90 (Fig. 6A; Supplementary Fig. S21), suggestive of a chronic inflammatory state, which was also implicated by the IFNγ transcriptome signature in neutrophils (Supplementary Fig. S19). The upregulation of these immunoregulatory factors was likely involved in the myeloid cell expansion, and may link our findings with previous reports of local inflammation in the skin of a different
imunologic substrain of K14HPV16 transgenic mice as well as patients with SCC (40–42). These results further suggested that the effects of local inflammation of the skin could be transmitted systemically to drive the expansion of myeloid cells, mediated by G-CSF and GM-CSF along with other factors.

**Myeloid cell depletion in K14HPV16 mice was too short-lived to rescue immune responses**

We next conducted a series of drug trials aiming to disrupt the systemic immunosuppression and enhance the antitumor immune response in K14HPV16/H2b mice. Namely, we tested the depleting antibodies anti-Ly6G, anti-Ly6C, and combination of anti-GR1, anti-GM-CSF, and anti-G-CSF, as well as chemotherapy treatments with 5-fluorouracil (43), gemcitabine (44), and a combination of carboplatin and paclitaxel (CarboTaxol; ref. 20). Although some of the treatments caused a transient decrease in certain myeloid cell populations (Supplementary Figs. S23–S27), their effects were usually short lasting and typically induced a compensatory increase in other myeloid populations. Ultimately, none of the treatments were capable of boosting the immune response to the E7 vaccination (Supplementary Figs. S23–S27).

**Effects of checkpoint blockade were masked by immunosuppressive cells in K14HPV16**

Having identified myeloid cells as the dominant immunosuppressive population in K14HPV16/H2b mice, we revisited the failure of ICB to boost the production of antigen-specific CD8+ T cells following vaccination with the NP-E7LP formulation. Notably, in an effort to improve upon the efficacy, we employed “next generation” isotype-switched versions, using both an anti-PD-1 that does not activate FC receptor functions (45), and an anti-CTLA4 that possesses Treg depleting activity (46). Antibody treatment was initiated at the same time as vaccination. Analysis of the immune response 9 days later showed neither an increase in E7-specific CD8+ T cells (Supplementary Fig. S28A and S28B) nor in cytokine production upon antibody treatment compared with the vaccine alone (Fig. 7; Supplementary Fig. S28C).

To determine if cytokine production by CD8+ T cells elicited in the vaccine plus ICB-treated K14-HPV16/H2b mice was intrinsically defective or repressed in paracrine fashion by the immunosuppressive cell milieu of the spleen, we isolated CD8+ T cells from treated mouse spleens, and performed *ex vivo* restimulation assays. We found that cytokine production was significantly higher in CD8+ T cells isolated from the spleens of HPV transgenic mice that received anti-PD-1 and anti-CTLA4 in combination with the NP-E7LP vaccine (Fig. 7). This result indicated that the addition of ICB to the therapeutic vaccination was indeed enhancing the CD8+ T-cell responses, resulting in superior cytokine production. However, such benefits were nullified and undetectable when CD8+ T cells were immersed in the total splenocyte cell milieu, as revealed when the CD8+ T cells were separated from other splenocytes. This was presumably due to the presence of immunosuppressive factors produced by myeloid cells that inhibited CD8+ T cells.

Similar results were obtained with CD8+ T cells isolated from mice treated with a combination of anti-GM-CSF, anti-G-CSF, and anti-GR1 (Supplementary Fig. S29). Namely, E7-specific CD8+ T cells isolated from mice that received the myeloid cell–targeting combination showed a significant increase in cytokine production compared with mice that only received the vaccine (Supplementary Fig. S29), despite the lack of measurable depletion at the observed timepoint (Supplementary Fig. S27); thus, we inferred transitory inhibition of their immune-inhibitory activity without myeloid cell depletion. Although we did not investigate the possibility of brief depletion of myeloid cell numbers at earlier timepoints, these results further support the conclusions that myeloid cells were suppressing immune responses in K14HPV16/H2b mice and the idea that sustained myeloid cell abrogation would improve antitumor immune responses.

**Discussion**

Despite the unprecedented successes of immunotherapies (9), a significant percentage of patients with cancer receive limited or no benefit from such treatments (11). Elucidating the mechanisms leading to the failure of current immunotherapies is a crucial step towards the development of more broadly efficacious therapeutic strategies. Myeloid cells have been observed in patients with cervical cancer (20, 22) but their importance has remained unclear.

In this study, we leveraged a genetically engineered, fully immunocompetent mouse model that phenocopies characteristics observed in patients with cervical cancer that may contribute to their poor responsiveness to tumor vaccines and ICB (18–22). By comparing K14HPV16/H2b mice with their nontransgenic FVB/N/H2b littermates and focusing on the lymphoid organs where immune responses are orchestrated rather than on the tumor microenvironment, we have uncovered an expansion in myeloid cells that were capable of suppressing both APCs and CD8+ T cells, abrogating both the effects of therapeutic vaccination as well as the synergistic effects of ICB, thereby rendering these therapeutic approaches ineffective.

Clinical trials involving therapeutic vaccination with E7 long peptides are showing little or no benefit (14, 16), much as we have observed in our mouse model. While a “solid-phase” NP-based formulation induced the development of detectable numbers of E7-specific CD8+ T...
cells in spleen and lymph node, their abundance was low, their ability to secrete inflammatory cytokines was severely impaired, and there was no appreciable accumulation proximal to or inside cervical carcinoma. In addition to direct CD8+ T-cell suppression, myeloid cell–mediated suppression of dendritic cell/APC activation is likely contributing to defective T-cell priming, evidenced by the inability of robustly upregulating a variety of costimulatory molecules on APCs. Although others have reported a role for Tregs in suppressing antitumor responses in other mouse models (23, 35), our analyses do not implicate these cells in the systemic immunosuppressive mechanism described herein, leading us to conclude that myeloid cells are the culprits.

Our results supported the hypothesis that neoplasia-induced systemic expansion of immunosuppressive myeloid cells can establish a first line of defense against efficacious tumor immunity (22). As such, these cells or their suppressive functions will need to be disrupted to allow for the generation of a strong and fully functional antitumor immune response. In addition, myeloid cells dominantly suppressed the beneficial effects that ICB has on CTL activity. Accumulation of myeloid cells has been reported for cervical and a wide variety of other cancers where they might be suspected to cause potent systemic immunosuppression (20, 22, 39, 47), potentially explaining the failure of immunotherapy in a certain patients.

Clinically feasible, specific, and effective treatments aimed at targeting myeloid cells are still lacking. Although certain chemotherapeutic agents have shown the ability to decrease myeloid cell numbers and produced promising results in combination with therapeutic vaccination (20), new myeloid cell–targeting strategies are clearly needed. The hyperexpansion of the systemic myeloid compartment observed in K14HPV16/H2b mice could render the condition particularly hard to counteract, it presents this model as an attractive platform to elucidate the details of the mechanisms responsible for the expansion and immunosuppression, thereby guiding the design of next-generation targeting approaches.

This study helped to clarify the significance of systemic myeloid cell–mediated immunosuppression and its link to the failure of immunotherapy (48). The abundance of immunosuppressive myeloid cells in both spleen and lymph nodes, and their ability to repress CTLs and DCs that were critical for the generation of a strong systemic antitumor response, helped to explain the poor immune responses to the HPV16 E7 neoantigens in our mouse model. Similar suppressive features have been reported for myeloid cells isolated from patients with HPV-related cancers (20, 22), encouraging the relevance of these findings and presenting clues as to why immunotherapy can be ineffective in patients with cervical cancer, and potentially in patients with other types of cancer displaying increased MDSCs (47, 49, 50) in spleen and lymph node. Targeting immunosuppressive myeloid cells may help to achieve successful immunotherapy in patients with cancer with systemic myeloid expansion.

Disclosure of Potential Conflicts of Interest
A.J. Korman has ownership interest (including patents) in Bristol-Myers Squibb. L.M. Coussens is a professor and chair at Oregon Health & Science University, a consultant for Cell Signaling Technology, an external advisory board member for Syndax Pharmaceuticals, and a scientific advisory board member for Carisma Therapeutics, Verseau Therapeutics, Zymeworks, and CytoMx Therapeutics, reports receiving a commercial research grant from Syndax Pharmaceuticals, reports receiving other commercial research support from Plexxicon, Acerta Pharma, Cell Signaling Technology, and NanoString Technologies, and is a consultant/advisory board member for Pharmacies, Inc. steering committee for PCYC-1137-CA, Cancer Research Institute (CRI), The V Foundation for Cancer Research, Cancer Research United Kingdom (CRUK) Early Detection (EDx) Research Committee, Starr Cancer Consortium, and Lustgarten Foundation for Pancreatic Cancer Research, Therapeutics Working Group. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

Acknowledgments
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References


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Myeloid Cells Orchestrate Systemic Immunosuppression, Impairing the Efficacy of Immunotherapy against HPV + Cancers

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