Combining T-cell Vaccination and Application of Agonistic Anti-GITR mAb (DTA-1) Induces Complete Eradication of HPV Oncogene Expressing Tumors in Mice

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Summary: We generated an adenovirus-based T-cell vaccine (Ad-p14) that reliably elicits T-cell responses to human papillomavirus (HPV) oncogenes of the 2 most common high-risk HPV serotypes. The artificial gene used to create the vaccine comprising 415 aa (1248 bp) was cloned by fusing 14 polymerase chain reaction fragments of HPV16 and HPV18 E6 and E7 oncogenes devoid of sequences with transforming potential. Although ensuring maximal biologic safety, the construct includes approximately 70% of the relevant T-cell epitopes. In a tumor model for cervical cancer (C3), therapeutic vaccination led to complete eradication in 100% of the mice. In a second model (TC1), it induced initial tumor mass reduction, but 90% of the animals showed delayed tumor progression. To further improve the therapeutic effect, vaccination was combined with systemic application of imiquimod, anti-CD4, α-interferon, or anti-GITR. Although adding α-interferon improved the therapeutic potential of Ad-p14 by 40%, the combination with anti-GITR resulted in complete and permanent eradication of all TC1 tumors. Ad-p14 has clinical potential for treating HPV-induced lesions, and the added effect of immune response modifiers stresses the importance of combined protocols for immunotherapy of malignant tumors.

Key Words: HPV, papillomavirus oncogenes, cervical cancer, therapeutic vaccine, adenovirus, GITR receptor

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INTRODUCTION

Incidence of Human Papillomavirus-induced Lesions

In Germany alone, about 500,000 women currently suffer from cervical intraepithelial neoplastic lesions caused by human papillomavirus (HPV) infections.[1,2] Owing to the elevated risk of progression to cervical cancer, about 50,000 affected women per year require surgery (conization).[3,4] After the first conization, about 50% of the patients have positive surgical margins and need additional treatment.[4] Prophylactic vaccines such as Gardasil (Merck) or Cervarix (GlaxoSmithKline) raise immunity against the 2 most common HPV subtypes (HPV16 and HPV18) but leave the 30% unprotected against other high-risk papillomaviruses.[5] Moreover, prophylactic vaccination does not influence the course of already established lesions.[5] Thus, regular screening programs for cervical lesions will remain indispensable, and it is imperative to develop reliable, safe, and less burdensome therapies.

HPV DNA can be detected in more than 99.7% of cervical cancers,[6] and HPV oncogenes are regularly expressed in high-grade CIN lesions.[6,7] The majority of earlier clinical trials to assess immunotherapy of cervical lesions focused on generating HPV E6/E7-specific $CD8^+$ T cells to eliminate HPV-infected cells.[8–17] Fusion proteins,[12,14,15,17] virus-based vaccines,[13,15] peptides,[10] and ex vivo stimulated antigen-presenting cells[9,16] have been applied in clinical trials. Despite convincing preclinical results, however, these agents mostly proved ineffective in clinical studies. Although HPV-specific cytotoxic T cells were regularly detected,[9,11–14,16,17] they failed to mediate a significant therapeutic effect. Like other human malignancies, HPV-induced lesions also harbor a set of local immune evasion mechanisms that modify the antigen presentation and reactivity of invading T cells.[18–22] Improving the clinical benefit of immunotherapy will involve overcoming local immune suppression. This study explored the benefit of combining T-cell vaccination with systemic application of different immune response modifiers. Imiquimod, a nucleoside analog of the imidazoquinoline family, is applied for topical treatment of genital warts. Local skin application induces an inflammatory response mediated through activation of toll-like receptors 7 and 8.[23] However, excessive inflammatory side effects limit the practicability of imiquimod for treating cervical lesions.[24] The second immune response modifier, explored in our study, was anti-CD4 mAb. The CD4 coreceptor is a very unspecific target structure, as CD4 is expressed on the majority of professional antigen-presenting cells. Noteworthy, however, is the evidence provided by preclinical studies that $CD4^+$ T-cell depletion can enhance the reactivity of cytotoxic T cells against tumor antigens, probably by sequestration of immunosuppressive $CD4^+$ $CD25^+FoxP3^+$ regulatory T cells.[25] Alpha-interferon is released from many different cell types after contact with viral or bacterial antigens and improves the recognition of foreign antigens and virus-infected cells by stimulating protein degradation and MHC-mediated presentation of foreign proteins.[26] During the late 1980s and early 1990s, interferons raised high expectations of potential benefits to be gained in the treatment of virus-induced neoplasms, such as CIN lesions

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TABLE 1. Sequence Motifs of HPV E6 and E7 Oncogenes Involved in Transformation of Mammalian Cells

Motif	aa Position in E6 HPV16 (HPV18)	Function
MFQ	8-10 (8-10)	Degradation of p53[27,28]
CxxC	37-40 (32-35)	Essential for structure formation (2 zinc fingers)[29,30]
	70-73 (65-68)	
	110-113 (105-108)	
	143-146 (138-141)	
CPEE	118-121 (118-121)	Telomerase activation[27]
RRETQL (V)	l53-158 (153-158)	Binding to PDZ domain containing cellular proteins[31–33]
	aa position in E7 HPV16 (18)	
LxCxE	22-26 (25-29)	Mediates Rb-binding and histone deacetylase activation, promotes proliferation[26]
CxxC	58-61 (63-66) 91-94 (98-101)	Essential for structure formation (zinc finger)
EDLL	80-83 (87-90)	Modifies function of cell cycle proteins (p21waf-1, p27Kip1, S4, M2-PK), stimulates aerobic glycolysis[34–36]

of the uterine cervix. Unfortunately, however, their effect as topical agents was mostly disappointing,[27–29] but they have been mostly explored as single-agent therapies and not in the context of a T-cell vaccination.

Another particularly effective switch for controlling T-cell reactivity in mice was identified by Simon Sakaguchi about 5 years ago. He developed an agonistic anti-GITR (glucocorticoid-induced TNFR-related protein) antibody whose systemic application induced a strong antitumor response.[30] The mode of action leading to tumor destruction is not entirely clear. The therapeutic effect was initially attributed to inactivation of murine regulatory T cells, but more recent findings suggest that direct stimulation of specific cytotoxic T cells might play a more important role in this context than the suppression of regulatory T cells.[31] The strong impact of GITR activation on the antitumor response in mice has raised hopes of cliical benefits in tumor therapy. Unfortunately, recent data suggest a different and, in part, an opposite physiological function of GITR signal pathway in humans.[32–34] However, the improved therapeutic effects achieved by combining vaccination and application of immune response modifiers demonstrate the need for expanded protocols to further optimize the antitumor response.

Constructing a Safe HPV E6/E7-based T-cell Vaccine

The HPV E6 and E7 oncogenes are potent mitogenic agents that interfere with cell cycle control, apoptosis, growth factor release, DNA repair, and genomic stability (Table 1).[35,36] The oncogenic properties of the E6 and E7 oncogenes are not restricted solely to full-length wild-type proteins. Peptide-binding studies and mutational analyses have revealed that native E6 and E7 proteins have multiple small protein-binding sites that interact with a large number of cellular proteins (Fig. 1, Table 1).[35–47] The integrity of wild-type oncoproteins is not required for protein binding. Small peptides containing binding motifs, such as LxCxE (aa 22 to 26 of HPV16-E7) or the C-terminal PDZ-binding domains RRETQL of E6 (aa 153 to 158 in HPV16-E6), are able to interfere with the Rb-associated histone deacetylase complex or the multiple PDZ domain containing tumor suppressor genes, such as MAGI-1 and SAP97/dlg.[43,47,48] In earlier studies, the mutagenic potential of antigenic oncoproteins was diminished by insertion and point mutations, gene fusions, and random, such as, rearrangement of gene fragments (so-called shuffling).[49–52] Despite modifications, all constructs described still harbor a nearly complete set of representative immunogenic fragments. In natural virus infections, viral DNA fragments are often stably integrated into the host genome and can be detected for many years to come.[53] Gene transfer studies have shown that local intramuscular application is not able to prevent a rapid systemic spread of DNA throughout the entire organism. Traces of DNA remain in the recipient for a long time, which makes safety aspects particularly important.[54,55] Standard transformation assays and mutagenicity tests in mice are not very

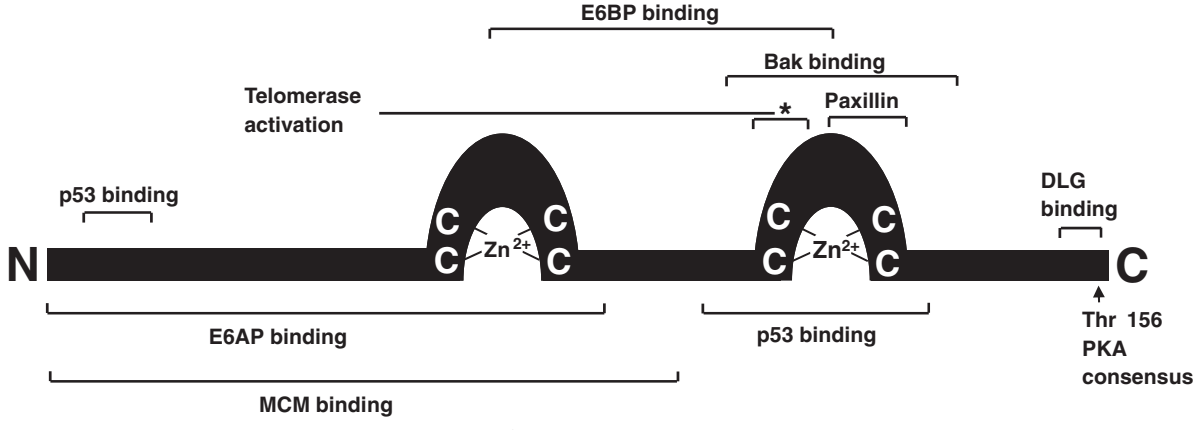

FIGURE 1. Binding sites for target proteins of HPV18 E6.[25]

sensitive and cannot provide information about subtle long-term effects on the cell cycle and genomic stability. We therefore conclude that maximum biologic safety can only be achieved by completely eliminating all suspicious sequence elements from the vaccine gene. In this study, we present a vaccine gene that provides maximum biologic safety, although still eliciting a strong and therapeutically effective T-cell response against tumors expressing HPV oncogenes.

MATERIALS AND METHODS

Construction of the Recombinant Vaccine Gene

Two plasmids encoding for the complimentary DNAs of HPV16 E6/E7 and HPV18 E6/E7 were kindly provided by Ethel-Michel de Villiers from the German Cancer Research Center in Heidelberg. Fourteen oncogene fragments, suitable for cloning into the recombinant vaccine gene, were amplified by polymerase chain reaction (PCR) and cloned into pSL1190 through adapted restriction sites. Expression of the recombinant gene was controlled by cytomegalie virus (CMV) promoter and CMV poly-A. The primer sets used for PCRs appear in Table 2.

All PCR fragments, intermediate products, and the final construct were sequenced to exclude mutations.

Epitope Prediction

MHC type-1 epitope prediction of the p14 vaccine gene was performed online with SYFPEITHI software contributed by the Institute for Cell Biology, BMI-Heidelberg, Germany (http://www.syfpeithi.de).

Transformation Assay

The p14 vaccine gene (*pCMV-p14*) was assessed for transformation activity by comparison with HPV oncogenes and control plasmids after stable transfection on NIH3T3 cells. NIH3T3 cells were seeded in 6-cm plates and cultured under standard conditions [Dulbecco's modified Eagle's medium (DMEM), 10% fetal calf serum (FCS), Pen/Strep]. After cells were grown to 50% confluence, 1 μg of selected plasmids, previously mixed with 0.1 μg of the selectable marker bearing plasmid pmaxFP-Green-N (Neo, AMAXA), was transfected by calcium phosphate coprecipitation. The following plasmids were tested separately in 3 independent trials: pCMVp14, expressing the p14 vaccine gene under control of a minimal CMV promoter and CMV-pA, pHPV16 E6/E7, expressing HPV16 E6 and E7 oncogenes, *pHPV18 E6/E7*, expressing HPV18 E6 and E7

TABLE 2. PCR Primer Sets Used for Amplification and Cloning of HPV16 and HPV18 E6 and E7 Oncogene Fragments

Fragments	Primer	Sequence
1. aa 62-79 in HPV16-E7	Nsp-16E7-P3	gga tcg ttc gaa cat gga ctc tac gct tcg gtt gtg
	Sal-16E7-P3	gtc acc gtc gac caa agt acg aat gtc tac gtg tg
2. aa 27-57 in HPV16-E7	Sal-16E7-P2	cgt cac gtc gac caa tta aat gac agc tca gag gag
	Bsp-16E7-P2	gct taa ggg ccc aaa ggt tac aat att gta atg ggc
3. aa 1-21 in HPV16-E7	Bsp-16E7-P1	gat cgt ggg ccc atg cat gga gat aca cct aca ttg c
	Mun-16E7-P1	gga gcc caa ttg atc agt tgt ctc tgg ttg caa atc taa c
4. aa 67-86 in HPV18-E7	Mun-18E7-VI	tga tga caa ttg aag tgt gaa gct aga att gag cta g
	Bgl-18E7-VI	gat gat aga tct gaa tgc tcg aag gtc gtc tgc tga g
5. aa 30-62 in HPV18-E7	Bgl-18E7-V	gta atg aga tct caa tta agc gac tca gag gaa gaa aac gat g
	Nhe-18E7-V	Tag gta gct agc caa cat tgt gtg acg ttg tgg ttc ggc
6. aa 1-24 in HPV18-E7	Nhe-18E7-IV	tga tga gct agc atg cat gga cct aag gca aca ttg c
	Asp-18E7-IV	Tag gat ggt acc gtc aac cgg aat ttc att ttg agg ctc
7. aa 122-142 in HPV16-E6	Asp-16E6-X	tga ggt acc aag caa aga cat ctg gac aaa aag c
	Bsp-16E6-X	Gtt tcc gga tcg acc ggt cca ccg acc cct tat att atg g
8. aa 74-109 in HPV16-E6	Bsp-16E6-IX	gta tcc gga tta aag ttt tat tct aaa att agt gag tat aga c
	Avr-16E6-IX	ggt cct agg cct aat taa caa atc aca caa cgg ttt gtt g
9. aa 41-69 in HPV16-E6	Avr-16E6-VIII	Tcg cct agg aag caa cag tta ctg cga cgt gag g
	Sac-16E6-VIII	tgg ccg cgg tac agc ata tgg att ccc atc tct ata tac tat gc
10. aa 12-36 in HPV16-E6	Sac-16E6-VII	gtc ccg cgg cca cag gag cga ccc aga aag tta cca cag
	Nru-16E6-VII	gtc tcg cga ttc taa tat tat atc atg tat agt tgt ttg cag c
11. aa 122-137 in HPV18-E6	Nru-18E6-XIV	gta tag tcg cga aat gaa aaa cga cga ttc cac aaa ata gc
	Xho-18E6-XIV	agt gta ctc gag ctg gcc tct ata gtg ccc agc
12. aa 69-104 in HPV18-E6	Xho-18E6-XIII	gag gta ctc gag ata gat ttt tat tct aga att aga gaa tta aga c
	Spe-18E6-XIII	gag tga act agt cct tat taa taa att gta taa ccc agt gtt ag
14. aa 36-64 in HPV18-E6	Spe-18E6-XII	gag gta act agt aag aca gta ttg gaa ctt aca gag gta ttt gaa ttt gc
	PmaCI-18E6-XII	gag gat cac gtg tgc agc atg cgg tat act gtc tct ata c
14. aa 7-31 in HPV18-E6	PmaCI-18E6-XI	gcg gag cac gtg cca aca cgg cga ccc tac aag cta cc
	Afl-18E6-XI	gag gtg ctt aag ggt tat ttc tat gtc ttg cag tga agt gtt c

oncogenes, *pEJras*, expressing the *Ha-ras* oncogene from the E J/T24 human bladder carcinoma cell line, and the empty expression vector pCMV-SL1, encoding the CMV promoter and CMV-polyA. Twenty-four hours after transfection, cells were cultured in selective medium (DMEM, 10% FCS, Pen/Strep, 0.5 mg/mL of G418) for 1 week. After selection, cells were trypsinated, and transfection efficacy (GFP expression) was assessed by flow cytometry. The cell density of the trypsinated cells was determined in a Neubauer chamber, and the cell solution was adjusted to 1000 cells/mL with DMEM. One milliliter of the cell solution (1000 cells) was mixed with 29.5 mL of 2X DMEM (13.48 g of Flow DMEM, 500 mL of distilled water, 3.7 g of bicarbonate, 20% FKS, 2X Pen/Strep, 4 mM glutamine, and 1 mg/mL of G418) and 29.5 mL of 0.3% soft agar previously heated to 45°C and filled into a T25 flask. After cooling in ice water for 15 minutes, the flasks were kept at temperatures below 37°C. Foci were counted after 3 weeks.

Generation and Preparation of Recombinant Adenovirus

To generate recombinant E1/E3-deleted replication-deficient adenovirus (Ad5), the p14 vaccine gene was inserted into an adenovirus transfer plasmid (pdE1sP1A), and cotransfection of 293 cells with a second plasmid encoding for the other requisite adenovirus genes (*BHG10*) was carried out according to the method of Graham.[56] After a second plaque purification, viruses were propagated in 293 cells and harvested 2 days after infection. Cells were lysed by sonication for 1 minute (Labsonic U, Braun, Melsungen, Germany), and viral particles were purified by 2 rounds of cesium chloride density gradient centrifugation. Cesium chloride was removed by gel filtration on Sephadex G-25 (Pharmacia, Uppsala, Sweden). Equilibration was performed with injection buffer, containing 3 mM KCl, 1 mM $MgCl_2$, 1 × phosphate buffered saline (PBS), and 10% glycerol. A 0.45-μm filter was used for sterile filtration (Schleicher und Schuell, FP 030/2, Dassel, Germany). The virus suspension was stored in aliquots at −78°C. Titration was carried out by endpoint dilution assays on 293 cells in 96-well plates. In addition, particles were counted, using the spectrophotometry technique described by Mittereder et al.[57] Biologic titers were expressed as infectious particles (IP) per milliliter and physical titers as particles/mL. Particle concentrations of adenoviral stock solutions ranged from 2 to 5×10^{12} particles/mL. The number of IP was one order of magnitude lower on the average.

Animal Studies

Animal procedures were performed according to the European Guidelines for Animal Studies after having been approved by the Institutional Animal Care Committee. Six-week-old C57BL6 mice were purchased from Charles River (Sulzfeld, Germany). C3 and TC1 tumor cells were kindly provided by T-C Wu (The Johns Hopkins Medical Institutions, Baltimore, Maryland). Cells were cultured under standard conditions (DMEM, 10% FCS, Pen/Strep); before transplantation, they were trypsinated, washed, resuspended in PBS, and adjusted to a density of 5×10^6 cells/mL.

Four groups of 10 mice each (n = 10) were set up to explore the prophylactic effect of vaccination with Ad-p14. On day 0, 20 animals received a single intramuscular injection of 1×10^{10} IP of Ad-p14 in 200 μL injection buffer into the right outer flank, whereas the other 20 mice received the same amount of Ad-lacZ control virus. Twenty-one days later, 10 animals in the control and 10 in the vaccination group were transplanted with 1×10^6 C3. In the other 2 groups, TC1 cells were subcutaneously injected into the left inner flank. Tumor growth was monitored by caliper measurements twice a week.

To explore the therapeutic potential of Ad-p14, 20 mice were transplanted with 1×10^6 C3 tumor cells and another 20 mice with 1×10^6 TC1 cells by subcutaneous injection into the left inner flank. Eight days after transplantation, when tumors had reached a size of 30 to 60 mm^3, mice were split into experimental and control groups (n = 10). Animals in the experimental groups received a single injection of 1×10^{10} IP of Ad-p14, whereas those in the control groups were treated with a single injection of Ad-lacZ virus. Tumor growth was monitored by caliper measurements twice a week.

Monitoring Immune Modulation

The therapeutic effect of additional measures was determined by a 3-step procedure. First, 7 groups of the 10 C57BL6 mice each were set up, and all animals were transplanted with 1×10^6 TC1 cells on day zero. The control group (n = 10) received only one Ad-lacZ injection 8 days after transplantation of TC1 cells. Animals in the anti-GITR group received a single intravenous injection of 200 μg of anti-GITR mAb (DTA-1, Pharmingen, Biosciences) 8 days after transplantation without earlier vaccination. Animals in the other groups received a single intramuscular injection of 1×10^{10} IP of Ad-p14 8 days after transplantation. The additional treatment was initiated another 8 days after vaccination (16 d after transplantation). One group of mice received an intramuscular injection of 1×10^5 IU of human α-interferon (Roferon; Roche-Pharma, Reinach, Switzerland) on days 8, 10, 12, 14, and 16 after vaccination. Animals in the Ad-p14 + anti-CD4 group received 100 μg of anti-CD4 (GK1.5, Pharmingen-Biosciences) on days 8, 11, and 14 after vaccination. In the Ad-p14 + anti-GITR group, 200 μg of anti-GITR Ab (DTA-1, Pharmingen-Biosciences) were systemically applied 8 days after vaccination. Animals treated with Ad-p14 plus imiquimod (R837, InvivoGen) received intratumoral injections of 100 μg of imiquimod in 200 μL of PBS on days 8, 11, and 14 after vaccination. Tumor growth was monitored by caliper measurements twice a week.

Detecting HPV-specific Regulatory T Cells

Mice were transplanted with 1×10^6 TC1 tumor cells on day zero. On day 8, PBS alone was given to group 1, 200 μ of DTA1/mouse to group 2, 1×10^{10} IP of Ad-LacZ control adenovirus to group 3, and 1×10^{10} Ad-p14 to group 4 and 5. Sixteen days after the transplantation, mice of group 5 were treated with 200 μg of DTA1 plus the vaccine. Peripheral blood leucocytes (PBLs) were isolated from mouse spleens on day 21. After washing twice in PBS, spleens were transferred into a MACS C Tube in 5 mL of RPMI media and treated in the MACS Cell Dissociater (Spleen Program 0101). Cell suspensions were then filtered through a cell culture filter. Cells were collected in RPMI/10% FCS. To detect functional HPV-specific regulatory T cells, PBLs were restimulated in vitro by coincubation with irradiated EL-4 cells, expressing HPV16E7 (E:T 1:20) in RPMI/10% FCS for 5 days.

TABLE 3. Sequence Fragments of HPV16 and HPV18 E6 and E7 Oncogenes Selected for Cloning of p14 Vaccine Gene

			Position in p14
	Position in HPV16 E7		
(1)	aa 1-21	MHGDTPTLHEYMLDLQPETTD	aa 55-75
(2)	aa 27-57	QLNDSSEEEDEIDGPAGQAEPDRAHYNIVTF	aa 22-52
(3)	aa 62-79	DSTLRLCVQSTHVDIRTL	aa 2-19
	Position in HPV18 E7		
(4)	aa 1-24	MHGPKATLQDIVLHLEPQNEIPVD	aa 135-158
(5)	aa 30-62	QLSDSEEENDEIDGVNHQHLPARRAEPQRHTML	aa 100-132
(6)	aa 67-86	KCEARIELVVESSADDLRAF	aa 78-97
	Position in HPV16 E6		
(7)	aa 12-36	PQERPRKLPQLCTELQTTIHDIILE	aa 253-277
(8)	aa 41-69	KQQLLRREVYDFAFRDLCIVYRDGNPYAV	aa 222-250
(9)	aa 74-109	LKFYSKISEYRHYCYSLYGTTLEQQYNKPLCDLLIR	aa 184-219
(10)	aa 122-142	KQRHLDKKQRFHNIRGRWTGR	aa 161-181
	Position in HPV18 E6		
(11)	aa 7-31	PTRRPYKLPDLCTELNTSLQDIEIT	aa 367-391
(12)	aa 36-64	KTVLELTEVFEFAFKDLFVVYRDSIPHAA	aa 336-364
(13)	aa 69-104	IDFYSRIRELRHYSDSVYGDTLEKLTNTGLYNLLIR	aa 298-333
(14)	aa 122-137	NEKRRFHKIAGHYRGQ	aa 280-295

Flow Cytometry Staining

T cells isolated from mouse spleen were stained with rat CD4-allophycocyanin and CD8-PE mAbs (BD Pharmingen). The ability of HPV-antigen-primed CD8 T cells to produce cytokines after specific restimulation was assessed by intracellular staining for interferon (IFN)-g, performed according to the manufacturer's instructions (BD Pharmingen). Immunosuppressive cytokine production of IL-10 (BD Pharmingen) by FoxP3-positive regulatory T cells (eBioscience) was also detected by intracellular FACS staining. PBLs were coincubated with HPV16E7-transfected EL-4 cells, mock-transfected EL-4 cells, or TC1 tumor cells at a responder/stimulator (R:S) ratio of 10:1 for 2 to 3 hours at 37°C. Golgi Plug (brefeldin A, BD Pharmingen) was then added to block cytokine secretion, and incubation was continued for 14 hours at 37°C. The lymphoid EL-4 cell line was derived from C57BL/6 mice; therefore these cells exhibited an equivalent MHC I repertoire. T cells were then stained with anti-CD8 PE and anti-CD4 APC, washed, permeabilized, and intracellularly stained with anti-FoxP3 fluorescein isothiocyanate and anti-IL-10-PE or anti-IFN-g fluorescein isothiocyanate. Flow cytometry was used to measure cytokine production by CD8/CD4 T cells.

Monitoring Tumor Growth After T-cell Transfer

Two groups of C57BL6 mice, each consisting of 3 animals (n = 3), were vaccinated with Ad-p14 (1×10^{10} IP) and Ad-lacZ (1×10^{10} IP), respectively. Eight days after vaccination, PBLs were isolated from mouse spleens, washed twice in PBS, and transferred into MACS C Tubes in 5 mL of RPMI media and treated in the MACS Cell Dissociater (Spleen Program 0101). Cell suspensions were then filtered through a cell culture filter. Cells were collected in RPMI, and 2×10^7 PBLs were transferred by intraperitoneal injection to C57BL6 mice before PBLs transfer transplanted with 1×10^6 C3 tumor cells. Tumor growth was monitored by caliper measurements in a 10-day interval.

RESULTS

Design and Cloning of the Vaccine Gene p14

HPV16 and HPV18 E6/E7 oncogenes encode altogether 519 amino acids. After eliminating all oncogenically active sequence motifs (Table 1), 14 DNA fragments coding for peptides (21 to 36 amino acids) were selected to clone the vaccine gene. To avoid random reformation of wild-type protein structures, DNA fragments were ordered and cloned inversely to wild-type sequences. The resultant vaccine gene encodes 415 amino acids; 364 are derived from HPV16 and HPV18 oncogenes and thus comprise 70.1% of the wild-type protein sequences (Table 3). Gene expression in the recombinant p14 vaccine gene is controlled by minimal CMV promoter and CMV polyA. The presence of preserved MHC class 1 epitopes was verified by Syfpeithi epitope prediction software (BMI-Heidelberg, Germany). A0201, A2402, A0101, and A0301 are the 4 most common MHC I alleles in Europeans, 87.5% of the people carrying at least one of them. HLA-A0201, the most common HLA class 1 allele, is present in about 50% of the population. Epitope analysis revealed that 7 of the 8 highest-affinity HPV16 and HPV18 E6 epitopes are still encoded in the recombinant p14 vaccine gene (Table 4).

Transformation activity of the construct was tested, using a standard transformation assay in NIH3T3 cells. HPV wild-type oncogenes and control plasmids (pras)

TABLE 4. A0201, A2402, A0101, and A0301 Are the Most Common MHC Class 1 Alleles in Europeans

	HPV16-E6	HPV18-E6
A 0201	1-1-1-1-1 (5/5)	1-1-1-0-0 (3/5)
A 2402	1-1-0-1-1 (4/5)	1-0-0-0-1 (2/5)
A 0101	1-0-1-0-1 (3/5)	0-1-1-0-0 (2/5)
A 0301	1-0-0-1-0 (2/5)	1-0-0-0-1 (2/5)

Eighty-seven percent of Whites express at least 1 of these 4 MHC-I complexes. Seven of 8 of high-affinity epitopes present in HPV16 and HPV18 E6 wild-type oncogenes are preserved in the recombinant p14 vaccine (1 = present, 0 = absent).

TABLE 5. Transformation Assay on NIH3T3 Cells

Vector	Colonies	Transfection Efficiency (1000 Cells) (%)	Transformation Frequency (%)
pCMV-p14	0	21	0
pHPV16E6/E7	138	19	66
pHPV18E6/E7	21	38	5.5
pras	121	39	31
pCMVSL (mock)	0	17	0

Although HPV16 and HPV18 E6/E7 and human ras showed substantial transformation potential (66%, 5.5%, and 31%, respectively), the vaccine gene CMV-p14 is free of any transformation activity.

showed substantial transformation activity. No residual activity was noted in the p14 vaccine gene (Table 5). To optimize in vivo gene delivery and gene expression, the p14 vaccine gene was inserted into a replication-deficient recombinant E1/E3-deleted adenovirus (Ad5).

Prophylactic and Therapeutic Potential of Ad-p14 in Mice

To analyze the effect of the recombinant adenoviral vaccine (Ad-p14) on tumor development, C57BL6 mice were vaccinated 3 weeks before the inoculation with C3 and TC1 tumor cells. C3 and TC1 cells are fibrosarcoma cell lines originally established by cotransfection of ras and HPV16 E6/E7 into murine fibroblasts.[50] These cell lines serve as models for tumors expressing HPV oncogenes. Vaccinating mice with Ad-p14, before tumor cell inoculation, prevented C3 and TC1 tumor outgrowth (n = 10) with 100% reliability. Animals in the control groups received a single injection of Ad-lacZ 3 weeks before tumor cell inoculation. Rapid tumor progression was seen in 80% of the C3 group and 100% of the TC1 group (Table 6).

The therapeutic potential of the vaccine was determined in the following experimental setting. After transplantation with C3 or TC1 cells, C57BL6 mice were vaccinated with Ad-p14 and Ad-LacZ 8 days later, when tumors had reached a size of 30 to 60 mm^3. During the first 8 days after vaccination, tumor growth did not differ between the Ad-p14 and Ad-LacZ groups. Within the next 2 weeks, tumors regressed completely in all Ad-p14-treated animals, whereas unaltered outgrowth was seen in the controls (Fig. 2). The TC1 group displayed an initial 50% to 80% tumor volume reduction. Some tumors were no longer visible or even palpable. However, 9 of 10 animals later showed tumor recurrence (Fig. 3).

To explore the reason for tumor recurrence in the TC1 group, we analyzed E6, E7, and MHC expression in progressive tumors before and after vaccination. No difference was detected in these parameters (data not shown). Moreover, repetitive application of Ad-p14 every third day after transplantation (starting on day 5 or day 8 after transplantation) failed to prevent tumor recurrence (data not shown).

TABLE 6. Application of 1×10^{10} Intraperitoneal Ad-p14 21 Days Before Transplantation of 1×10^6 C3 and TC1 Tumor Cells Reliably Protected Mice From Tumor Growth (0/10), Whereas in Both Control Groups Transplantation of C3 and TC1 Cells Led to Rapid Outgrow of Tumors (Buffer Only: 10/10; Ad-lacZ 8/10)

	Buffer	Ad-lacZ	Ad-p14
C3	10/10	8/10	0/10
TC1	10/10	0/10	0/10

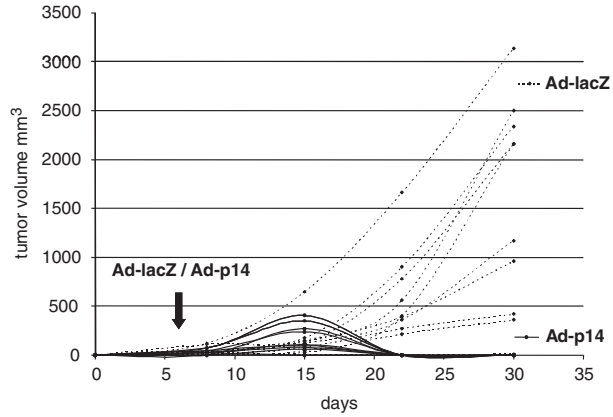

FIGURE 2. Treating mice (n = 10) 8 days after inoculation of C3 tumor cells (tumor size of 30 to 60 mm^3) by single intramuscular application of Ad-p14 (1×10^{10} intraperitoneally) induced complete and permanent tumor regression (observation period 200 d). No effect on tumor growth was seen after application of control virus (Ad-lacZ).

Immune Modifiers

As we were not able to cure mice with TC1 tumors by vaccination alone, we explored the therapeutic value of additional immune modifiers. Mice were divided into 7 groups (n = 10) and received different agents after vaccination with Ad-p14. Three intratumoral injections of 100 μg of imiquimod after vaccination with Ad-p14 had no effect on tumor growth, and cure rates were comparable to those in the control group, receiving Ad-p14 alone (Fig. 4). Little benefit was derived from treatment with 3 injections of anti-CD4 on days 8, 11, and 14 after vaccination (cure rate 20%) (Fig. 4). Additional application of human α-interferon (Roferon) on days 8, 10, 12, 14, and 16 after transplantation increased the

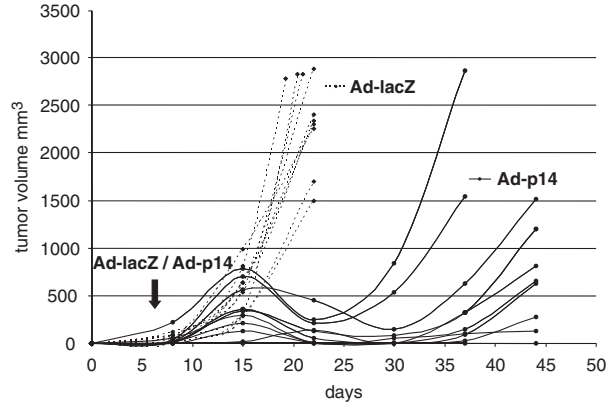

FIGURE 3. Eight days after inoculation of TC1 tumor cells, mice were vaccinated by single intramuscular application of Ad-p14 (1×10^{10} intraperitoneally). Initial reduction of tumor mass by 50% to 80% was seen in all mice of the treatment group (n = 10) but the therapeutic effect was only transient and tumor recurrence was observed in 9 of 10 animals.

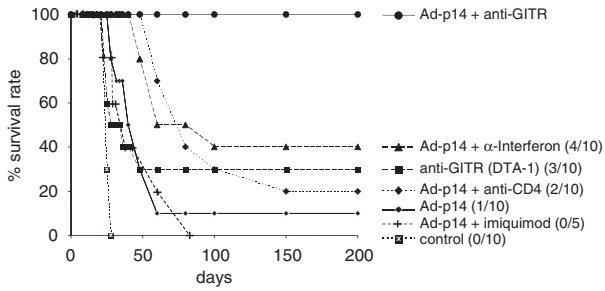

FIGURE 4. To improve the therapeutic effect of Ad-p14, vaccination was combined with the application of different immune modifiers. Immune modifiers were supplied 8 days after vaccination. No additional therapeutic effect was seen by combining imiquimod or and anti-CD4 treatment with Ad-p14 vaccination, whereas the supplemental application of α-interferon cured 4 of 10 animals. By fare, the most beneficial result was achieved by combining Ad-p14 and single systemic application of 100 μg anti-GITR mAb (DTA-1) 8 days after vaccination. This protocol induced complete and permanent regression of all TC1 tumors (cure rate 10/10). Applying the anti-GITR mAb (DTA-1) as single agent (without prior vaccination) led to remission of 3 tumors (cure rate 3/10).

cure rate to 40% (Fig. 4). The most impressive result was achieved by applying the anti-GITR Ab (DTA-1) only once 8 days after vaccination with Ad-p14. All animals (100%), treated with a combination of Ad-p14 and anti-GITR, showed complete and permanent tumor clearance (Fig. 4). Applying anti-GITR alone without earlier vaccination cured 30% of the animals.

T-cell–dependent Tumor Eradication

The role of cytotoxic T cells in tumor eradication and the additional therapeutic effect of anti-GITR mAb was explored on isolated murine PBLs. Eight days after transplantation of HPV16 E6/7 expressing TC1 cells, mice were split into 5 groups and treated with single intramuscular injection of PBS, anti-GITR mAb, Ad-LacZ, Ad-p14, and Ad-p14 plus anti-GITR mAb, respectively. Twenty-one days after tumor cell transplantation, PBLs were isolated from spleens, and tumors were collected for histologic staining. The density of $CD4^+/FoxP3^+$ T_{regs} in peripheral blood was determined by flowcytometrical analysis. No significant difference between treatment and control groups and, especially, no effect of systemic anti-GITR treatment on peripheral T_{reg} count was determined (data not shown).

The specificity of vaccine-induced cytotoxic T cells and the immunosuppressive effect of regulatory T cells was determined in T-cell assays by flowcytometrical analysis. Before the cytokine staining (γIFN), PBLs were restimulated in vitro by HPV16E7-expressing target cells (murine lymphoid EL-4 cells). For induction of cytokine secretion, PBLs were coincubated with 3 different target cells: EL-4 cells (mock), EL-4 HPV16E7 cells (positive control), and TC1 tumor cells. The amount of HPV-specific cytotoxic T cells, induced by vaccination, was determined by staining of $CD8^+$/γIFN T cells (Figs. 5A, B). Although no HPV-specific $CD8^+$ T cells were determined in both control groups (PBS, Ad-lacZ treatment), a significant amount of γIFN-secreting $CD8^+$ T cells was found in mice, receiving Ad-p14 vaccine (Figs. 5A, B). Combining therapeutic vaccination with Ad-p14 with systemic application of anti-GITR mAb further stimulated the specific T-cell response.

Analyzing the suppressive potential of circulating regulatory T cells ($FoxP3^+$/IL-10) in mice did not reveal a significant difference between treatment and control groups. Especially, after anti-GITR mAb treatment, no difference in the immunosuppressive status of peripheral T_{regs} was recognized (data not shown).

To confirm the specific role of cytotoxic T cells in tumor destruction, PBLs isolated from spleens of mice previously vaccinated with Ad-p14 or Ad-lacZ, respectively, were transferred to naive C3 tumor-bearing mice. All the animals receiving PBLs from Ad-p14-vaccinated mice, showed substantial tumor regression, whereas animals in the control group (Ad-lacZ) showed unaltered tumor growth (Fig. 6).

DISCUSSION

The design of a recombinant DNA vaccine, derived from multifunctional oncogenes, such as HPV E6 and E7, requires very high safety standards, as traces of DNA may spread after inoculation and may then remain in the recipient for a long time. Maximum biologic security can only be achieved by eliminating all suspicious sequence elements. As papillomavirus oncogene functions have been studied intensively, there is a large body of literature on critical sequence elements.[35–48] These studies demonstrate that functional properties of E6 and E7 are not necessarily dependent on the integrity of the wild-type protein. Even small peptide motifs, such as LxCxE or the PDZ-binding domains RRETQL at the C-terminus of E6, were found to be functionally active and capable of interfering with the cell cycle.[43,47,48] This study shows that it is possible to design a DNA vaccine that is devoid of all suspicious elements but still codes for most of the common high-affinity T-cell epitopes and evokes a strong specific T-cell response in vivo.

E6/E7-expressing C3 tumors were completely and permanently rejected after a single application of Ad-p14 vaccine in mice. In a second tumor model expressing HPV oncogenes (TC1), vaccination induced only partial tumor remission. Increasing the dose or applying the vaccine, repetitively, did not improve the outcome. However, low vaccination-induced anti-TC1 tumor responsiveness makes it possible to test the effect of additionally applying immune modifiers. This study applied imiquimod, anti-CD4, α-interferon, and anti-GITR 7 days after vaccination with Ad-p14. The idea behind the delayed application was to optimize T-cell reactivity during the effector rather than the induction phase of the T-cell response. Although anti-CD4 and imiquimod mostly failed to mediate an additional therapeutic effect, a cure was achieved in 40% of the mice by combining Ad-p14 and α-interferon treatment, and in 100%, by combining Ad-p14 and a single dose of anti-GITR mAb (DTA-1). In previous clinical trials, interferon, used as single-agent therapy, failed to have a beneficial effect on neoplastic lesions.[27–29] The outcome of our study suggests that biologic properties of α-interferon, involved in antigen presentation, could be used more efficiently in combination with a reactive T-cell response. Alpha-interferon (Roferon A) is routinely used to treat chronic hepatitis B and C, which would facilitate its utilization as an adjuvant for T-cell vaccination against HPV-induced lesions.

By far the most impressive result in our study was achieved by combining vaccination and treatment with anti-GITR mAb (DTA-1), which led to complete and permanent

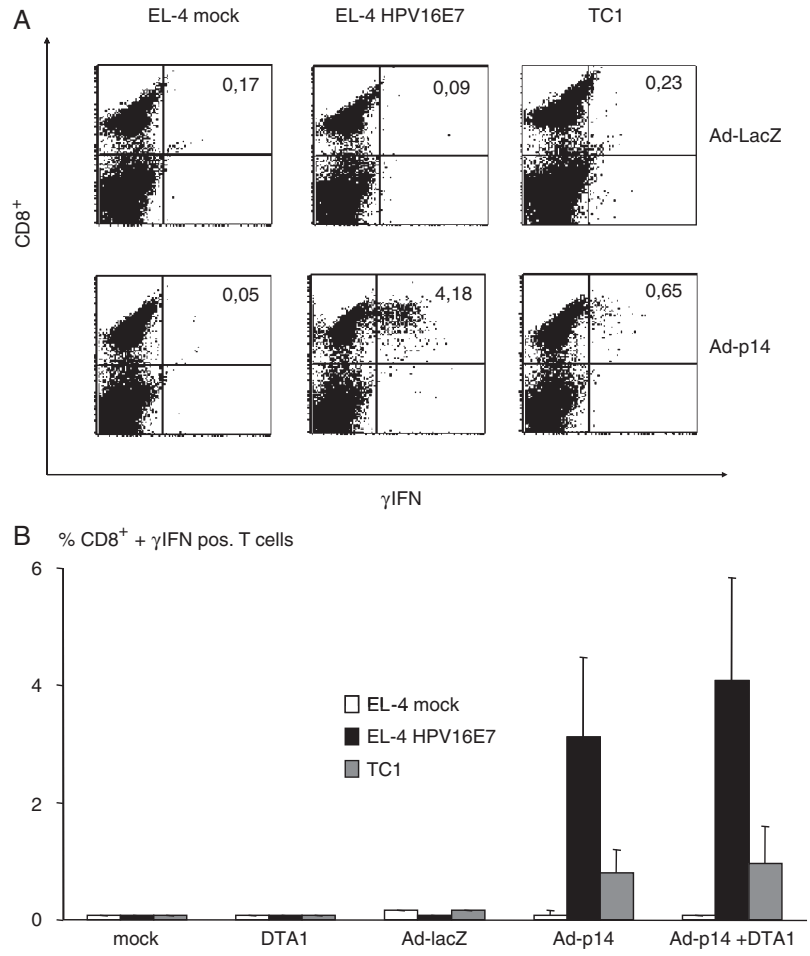

FIGURE 5. CD8+/γIFN FACS staining of isolated and restimulated peripheral blood leucocytes (PBLs). Cytokine secretion was induced by coincubation with HPV16 E7 expressing EL-4 cells [positive (pos.) control], naive EL-4 cells (mock), and HPV16E7 expressing TC1 tumor cells. A, Flow cytometric dot blots of CD8+/γIFN staining. No γIFN secretion was detected in PBLs of Ad-lacZ-treated mice (upper blots) whereas staining of PBLs of mice treated with Ad-p14 revealed a high frequency of CD8+-positive γIFN-secreting T cells (results of 3 independent measurements). B, Percentage of CD8+/γIFN-positive T cells.

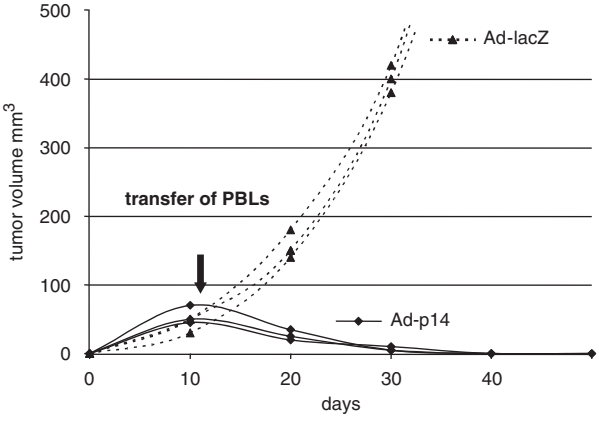

FIGURE 6. Tumor regression is seen after transfer of vaccine-primed peripheral blood leucocytes (PBLs) to naive C3 tumor bearing mice (n = 3). A fast outgrowth of tumors was monitored in mice treated with Ad-LacZ-primed PBLs.

remission of all TC1 tumors. Our study is the first to report an anti-GITR effect in a tumor model, expressing HPV oncogenes, but the remarkable antitumor response induced by DTA-1 has already been observed in other murine tumor models.[30] Abrogating the suppressive effect of regulatory T cells (T_{regs}) was initially assumed to be the most important mechanism of action,[30] but desensitizing $CD8^+$ effector T cells against inhibitory signals seems to have a greater impact on the therapeutic effect.[31] High hopes have been attached to the effect of GITR stimulation in humans, but controversial data over the last 3 years have left an ambiguous picture about GITR function in humans.[32–34] GITR activation protects human, and murine $CD8^+$ T cells from immunosuppressive signals, thus, keeping them in an activated state for a longer period of time.[58–60] Although murine T_{regs} lower their immunosuppressive potential after GITR activation, the suppressive state of human T_{regs} remains unaltered.[32–34,58–60] The influence of GITR on human NK cells is discussed controversially.[33,61] Stimulatory and inhibitory effects on human NK cells have been reported.

In addition, GITR ligand is apparently expressed by human tumors and seems to be involved in apoptosis of NK cells.[34] However, GITR remains a particularly interesting research target along with other members of the TNF receptor family, such as OX40 and 41BB, that are intimately involved in regulating T-cell functions. Among the most important conclusions to be drawn from our study is that monoagent T-cell vaccination has limitations. Improving the benefit will require combination protocols and the inclusion of immune modifiers that affect the physiological course of the T-cell response and require a correctly timed application.

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