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Monoclonal Antibodies Against Human Papillomavirus E6 and E7 Oncoproteins Inhibit Tumor Growth in Experimental Cervical Cancer

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Abstract

Nearly all cases of cervical cancer are initiated by persistent infection with high-risk strains of human papillomavirus (hr-HPV). When hr-HPV integrates into the host genome, the constitutive expression of oncogenic HPV proteins E6 and E7 function to disrupt p53 and retinoblastoma regulation of cell cycle, respectively, to favor malignant transformation. HPV E6 and E7 are oncogenes found in over 99% of cervical cancer, they are also expressed in pre-neoplastic stages making these viral oncoproteins attractive therapeutic targets. Monoclonal antibodies (mAbs) represent a novel potential approach against the actions of hr-HPV E6 and E7 oncoproteins. In this report, we describe the utilization of anti-HPV E6 and HPV E7 mAbs in an experimental murine model of human cervical cancer tumors. We used differential dosing strategies of mAbs C1P5 (anti-HPV 16 E6) and TVG701Y (anti-HPV E7) administered via intraperitoneal or intratumoral injections. We compared mAbs to the action of chemotherapeutic agent Cisplatin and demonstrated the capacity of mAbs to significantly inhibit tumor growth. Furthermore, we investigated the contribution of the immune system and found increased complement deposition in both C1P5 and TVG701Y treated tumors compared to irrelevant mAb therapy. Taken together, the results suggest that anti-HPV E6 and E7 mAbs exert inhibition of tumor growth in a viral-specific manner and stimulate an immune response that could be exploited for an additional treatment options for patients.

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Introduction

Cervical cancer remains the third leading cause of cancer worldwide claiming over 260,000 lives annually [1]. It is well established that human papillomaviruses (HPV) are the etiologic agent of nearly all-cervical cancers with HPV 16 and 18 are the most common of the HPV found in 70% cancers [2,3]. During the viral life cycle, integration of HPV into the host DNA occurs through an unknown mechanism [4]. At this critical point, transcriptional control by HPV E2 is lost and the overexpression of HPV E6 and E7 leads to a multi-step process of carcinogenesis [5–7]. Cervical cancer arises through four steps of HPV establishing residence in normal cervical tissues; 1) HPV transmission, 2) viral persistence, 3) progression of persistently infected cells, 4) pre-cancer to the final stage of invasive cervical cancer [8]. Along with other cellular events and proteins, HPV E6 and E7 begin to deregulate p53 and retinoblastoma (pRb) pathways, respectively, and instigate additional mechanisms such as genomic instability, deregulation of cellular genes that control the G2/M phase transition [4,9,10]. Without the actions of HPV E6 and E7, malignant transformation would not occur, thus these oncogenes are ideal targets for therapy [9,11].

While developing the approach of radioimmunotherapy (RIT) against HPV E6 and E7 oncoproteins we consistently observed the pronounced inhibition of tumor growth by unlabeled mAbs [12–14]. However, after noting significant tumor growth inhibition of a single intraperitoneal (IP) injection of anti-HPV E6 mAb (C1P5, Abcam Inc.) and anti-HPV E7 mAb (TVG701Y, Abcam Inc.) we began to consider the possibility of C1P5 or TVG701Y monotherapy. We hypothesized that because of the cellular turnover that occurs as solid cervical cancer tumors grow, HPV E6 and E7 oncogenes were made accessible to the antibody via necrosis [13–15]. Guided by our findings, we sought to test serial dosing strategies to evaluate the impact on tumor growth inhibition and analyze the host-tumor immune response to the treatment. In this report we test the impact of singular or combinatorial strategies of treatment with C1P5 and TVG701Y on tumor growth inhibition in experimental cervical cancer tumors. After employing different treatment strategies, we evaluate the potential additive or synergistic effects and quantify the contribution of the immune system.

Materials and Methods

Cell Lines

Cervical carcinoma cell lines, SiHa and CaSki (American Type Culture Collection, University Blvd, VA, Manassas) were grown in monolayers cultures at 37 °C in humidified atmosphere with 5% CO_2. Both Caski and SiHa were grown in RPMI-1640 cell culture media containing 10% fetal bovine serum (Sigma Chemical Company, St. Louis, MO) and 1% penicillin–streptomycin solution (Sigma, penicillin, 10,0000 U; Streptomycin, 10 mg/ml). CasKi and SiHa cell lines are hr-HPV 16 positive cervical cancers that express both HPV E6 and HPV E7 oncoproteins; CaSki (HPV 16 integrated virus) and SiHa (HPV 16 both integrated and episomal virus).

Antibodies and Reagents

C1P5 (Abcam Inc. ab70), anti-HPV16E6 + HPV18E6 antibody is a murine derived IgG1 isotype and TVG701Y (Abcam Inc. ab90) anti-HPV 16 E7 is a murine derived IgG2a istoype. Non-specific antibody control mAbs were identified as: MOPC-21 (AbCam Inc., ab18443) is a mouse IgG1 of unknown origin as well as chTNT3, an intracellular target against histone, humanized IgG TNT3 that binds to single-strand DNA, a kind gift from Dr. Alan Epstein, University of Southern California). Cisplatin attained from APP Pharmaceuticals, Fresenius Kabi USA, LLC.

Experimental CasKi Tumor Models

All studies described were carried out in accordance with the guidelines of the Albert Einstein College of Medicine Institute for Animal Studies. Six to eight-week-old female athymic balb/c nude mice were purchased from Charles River Laboratories (Malvern, PA) and maintained in pathogen-free conditions. Experimental tumors were developed by injection with 8 x 10^6 CasKi cells mixed with 100 µl of BD Matrigel™ Basement Membrane Matrix (BD Biosciences) implanted subcutaneously into the right flank of balb/c nude mice. After approximately 2 weeks post-injection of CasKi cells, tumors grew to 3–5 mm in diameter and afterwards randomized into respective treatment groups of five.

Mice were then individually tagged using an ear punch mechanism to identify mice in each treatment group. All mice were observed until the end of the experimental period. No mice died as a result of treatment, sickness, or unrelated causes. Electronic Calipers (instrument type) were used to measure tumors in the x, y, and z-axes. To account for the subcutaneous component, total volume was calculated in centimeters (mm^3) according to the equation: Volume = (2/3) * 3.14 (x * y * z). At the completion of the study, mice were sacrificed using $C0_2$ euthanasia and cervical neck dislocation prior to removing tumors from the flank of nude mice. Tumors were immediately embedded in paraffin according to standard protocol. Mice in the untreated control group were sacrificed prior to the end of the study period if tumors grew larger than $1cm^3$ or if tumors began to erode through the skin.

a) Pilot evaluation of dose-escalation of C1P5 treatment. Three groups of five mice bearing CaSki derived tumors were selected for study. A single intraperitoneal (IP) injection of 3 µg/ml C1P5 mAb versus 15 µg/ml C1P5 mAb per 20 g mouse was administered on Day 0 of the 18-day observation period. Tumors were measured every 3 days and volume calculated as previously described. Taking the average measurements of all mice per group we performed 2-way ANOVA evaluated comparison to untreated control. *P* value set at 0.05 to determine significance.

b) Serial dose of C1P5 dosing regimen compared to cisplatin. Mice were randomized into groups of five and treated with IP treatment on days 1–8 of the study period as described in Table 1. During the 49-day observation period tumors were measured and volume calculated. At the end of the observation period, mice were sacrificed, tumors surgically removed and paraffin embedded.

c) Therapeutic impact of intratumoral combinatorial C1P5 and TVG701Y strategies. Mice were randomized into groups of five and treated with 15 µg/ml C1P5 intratumorally (IT), or 15 µg/ml TVG701Y IT, or 7.5 µg/ml C1P5 IP followed by 7.5 µg/ml TVG701Y IP 1 hour later, or 7.5 µg/ml TVG701Y IP followed by 7.5 µg/ml C1P5 IP 1 hr. later, or 15 µg/ml isotype matching control MOPC 21 IP, or Cisplatin (three 50 µg doses on days 0, 4 and 8 after treatment). Untreated controls were administered phosphate buffered saline (PBS) IP or left untreated. During the 24-day observation period tumors were measured as described above.

Immunohistochemical (IHC) Analyses

At the end of the observation period in all murine models, mice were humanely sacrificed, tumors surgically removed, and paraffin

embedded. All tissues blocks were stained with hematoxylin and eosin (H&E) and independently evaluated and scored by two investigators.

H&E Baseline Characteristics

At the end of the study period, procured tumors were cystic with a firm consistency to the outward shell and fluctuant centrally. In some cases, surrounding skin and underlying muscle were excised to maintain intactness of the tumor. Baseline characteristics of the tumors on IHC H and E staining evaluated the ratio and location of live cells versus necrosis. Percent necrosis was identified on H&E slides from each tumor group. Using 20x magnification, 4 areas were evaluated to estimate the overall percent necrosis. Regions of necrosis are identified of areas of cellular clearing stained pink compared to the areas of viable intact tumor populations. Independent assessments from pathology reviewers were attained and the percentage reported as the average of two observations.

Evaluation of complement deposition

Paraffin embedded tumors are representative of deposition after 24 days after treatment. In this experiment, an auto-stainer was used and manufacturers protocol briefly is as follows: Deparaffinization process, followed by 48 minutes in CC1 cell conditioners (CC1 is a tris-EDTA based antigen retrieval solution) Use of inhibitor CM. Primary antibody use of ab200999@ 1:1000 for 32 minutes at 37C. Multimer − use was Ventana (Roche) Omni-mAb anti rabbit HRP #760−4311 for 20 minutes and counterstain was performed with DAB/hematoxylin. Complement deposition was assessed using murine C3 mAb (Abcam Inc.) at a dilution of 1:1000 using TRIS/EDTA antigen retrieval. The positive control used was human kidney from a lupus patient.

Statistical Analysis

The differences between tumor sizes measure in the control and treatment groups were analyzed using Graph Pad Prism Software two-way ANOVA. The differences were considered statistically significant when P values were <0.05.

Results

In Vivo Delivery of Differential Dose Strategies of C1P5

We found that both doses significantly ($P = .02$) retarded the tumor growth in comparison with untreated controls. (Figure 1) There was no statistically significant difference in tumor size between 3 µg and 15 µg C1P5 dose strategies. As no difference was observed between 3 and 15 µg doses, we chose to use 15 µg dose as it is closer to the doses of unlabeled or radiolabeled antibodies to E6 and E7 which we utilized in our previous research [12,13]. No mice in any treatment group died or had any apparent side effects. This data supported the hypothesis that C1P5 inhibits tumor growth and should be studied further as a "stand-alone" therapeutic modality using 15-µg dose. Therefore, all subsequent studies of mAbs utilized this dose.

CasKi derived experimental tumors response to C1P5 and TVG701Y.

All anti-HPV E6 and E7 oncogene treatment strategies exhibited consistent suppression of tumor growth throughout the 24-day observation period (Figure 5). Although combination of treatment strategies of C1P5 and TVG701Y was equally effective, when compared to independent C1P5 and TVG701Y treatment no increased inhibition of tumor growth was noted. As expected tumors in the Cisplatin treated group showed significant tumor growth inhibition throughout the study period. A non-specific isotype control mAb, MOPC21, was used as a negative control and demonstrated a trend toward decreased tumor growth compared to untreated controls, however this was not statistically significant.

Severity of necrosis depends on number of C1P5 dose administered.

In our initial investigations, we compared C1P5 treatment to that of Cisplatin, an alkylating agent that intercalates DNA and is used in the treatment of patients with cervical cancer as a radiation sensitizer. The direct comparison of the ability of C1P5 to induce apoptosis like Cisplatin is critical. To observe the dose response of unlabeled mAb therapy, the mice were randomized into groups of five and treated with 1, 2, or 3 doses of 15 µg C1P5 or 3 doses of 50 µg Cisplatin or left untreated. No statistical significant difference in tumor growth was noted between 1, 2, or 3 doses of C1P5. Encouragingly Cisplatin and C1P5 therapy were noted to have equivalent impact on tumor growth. When compared to untreated control tumors, all treatment groups exhibited statistically significant inhibition (Figure 3).

When tumors were removed after the study period, differential composition of tumors was noted: both C1P5 and Cisplatin were mostly fluid-filled cysts without solid components within the tumor mass whereas untreated controls were solid and firm. We surmise that in this xenograft model where blood supply is limited, the visible difference in size of tumor may be negligible between doses given. While there was no significant change in tumor volume between treatment regimes we observed striking differences in H&E staining of tumors demonstrated that the number of doses of C1P5 correlated with increasing percentage of necrotic cells: 1 dose − 30%, 2 doses − 60% and 3 doses 85% (Figure 4).

C1P5 Stimulates Complement Deposition

To gain insight into the role of antibody mediated tumor growth inhibition, complement deposition was assessed on tumors procured on day 5 after intraperitoneal injection using IHC (Figure 2). C1P5 treated group demonstrated enhanced deposition compared to untreated controls. Tumors treated with irrelevant isotype control murine IgG1 demonstrated some complement deposition but

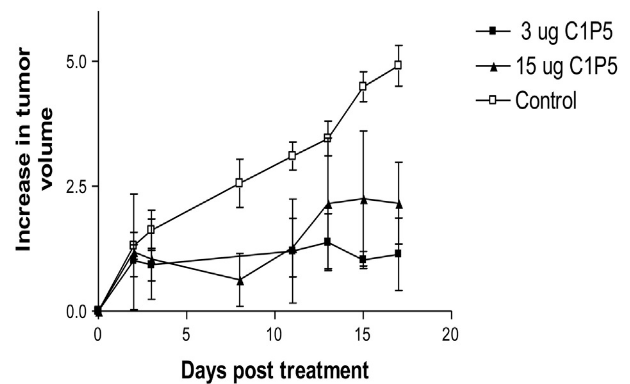


Figure 1. Pilot evaluation of dose-escalation of C1P5 treatment. Tumor growth curve of athymic nude mice bearing CasKi tumors treated with a single dose of intraperitoneal injections of 3 µg/ml or 15 µg/ml C1P5. Change in tumor volume over time among treatment groups was compared with untreated CasKi control tumors. Tumor volume measured over the 18-days observation period post treatment.

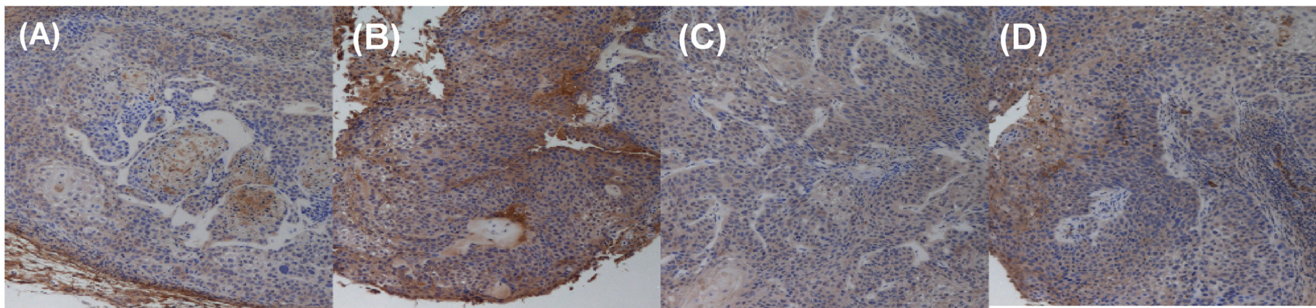


Figure 2. Activation of complement pathway enhanced by C1P5 administration. Experimental tumors procured on day five after mAb administration (Figure 1) C3 staining noted by brown staining indicates the complement activation. Original magnification 10×. A) Tumors treated with murine isotype control MOPC21; B) Tumors treated with C1P5 monoclonal antibodies; C) Tumors treated with humanized IgG TNT3; D) Untreated tumors.

amount of deposition was less than that of C1P5 treated tumors. Humanized TNT3 treated mice did not demonstrate complement deposition suggesting that deposition of the complement in a murine model might require murine Fc region on the mAb.

Accumulation of complement in tumor tissue causes elimination of cancer cells by forming membrane attack complex and opsonization of the target cells and is considered activation of complement pathways in order to facilitate immune surveillance [16,17]. A potential mechanism is that binding of complement proteins also allow the apoptotic and necrotic cells to be recognized by macrophages leading to engulfment of dying cells [18].

Discussion

In effort to answer the urgent call to develop options in the treatment of cervical cancer, we have developed an innovative approach for the treatment of HPV-induced cervical cancer that is highly effective, specific, with low toxicity [12–15,19]. The association of hr-HPV genotypes to cervical cancer has been universally recognized for several decades. Despite significant advances in understanding the mechanism of HPV-infection and the causative role in cervical cancer [20,21], there are no HPV-specific therapies utilized in clinical practice outside of a clinical trial [22–24].

When invasive cancer is diagnosed in the early stage - it is largely curable with surgery and/or radiation with chemotherapy [25]. However, about 30% of patients will have a recurrence of cervical cancer where the prognosis is poor, with one-year survival rates between 15–20% [24]. Many chemotherapeutic approaches have been tried in the clinic in patients with recurrent, persistent or metastatic cervical cancer with limited efficacy [26]. To date, the most significant improvement in overall survival is gained with the addition of bevacizumab, an anti-angiogenesis drug, to toptecan plus paclitaxel chemotherapy regimens [27]. While clinical benefit is apparent, in overall survival advantage of 3.7 months, this highly toxic regimen limits successful treatment in many cases. Therefore additional approaches must be continually developed.

Mostly molecular targets in clinical usage have been tested in cervical cancer non-specifically and target drugs evaluated for therapeutic potential without knowledge of driver mutations. In clinical trials, small inhibitory RNA (siRNA) based therapies in clinical trials since 2004 however currently there are no FDA

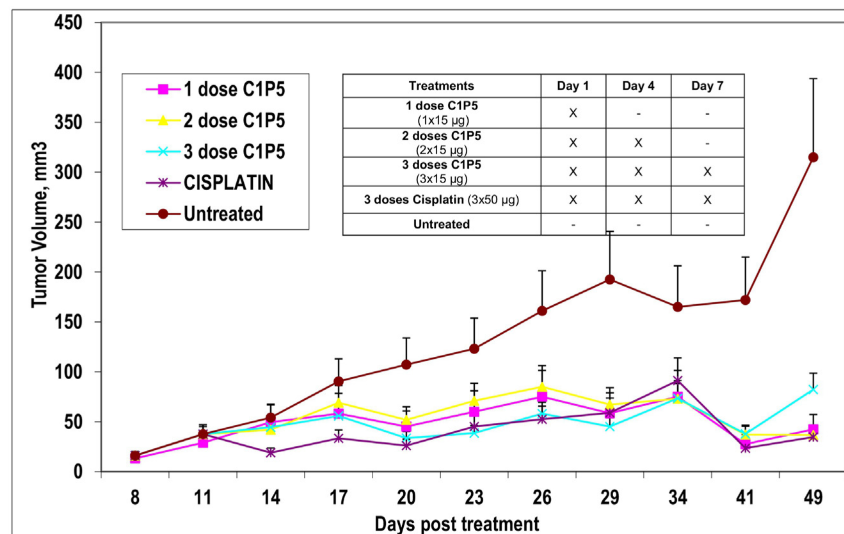


Figure 3. Serial Doses of C1P5 demonstrates comparable efficacy to Cisplatin treatment. Dosing strategies administered during the first 7 days of treatment. After intraperitoneal injections of 15 μg C1P5 and single dose of 50 μg Cisplatin compared to untreated groups. Tumor volume ($V_2 - V_0/V_0$) was measured serially throughout the 49 days of observation period. All doses of C1P5 and Cisplatin showed similar efficacy and significant decrease of tumor volume ($P < .05$).

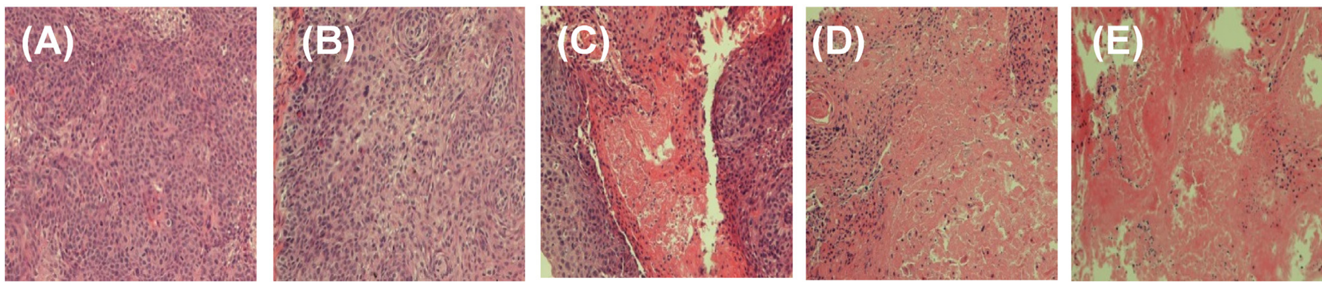


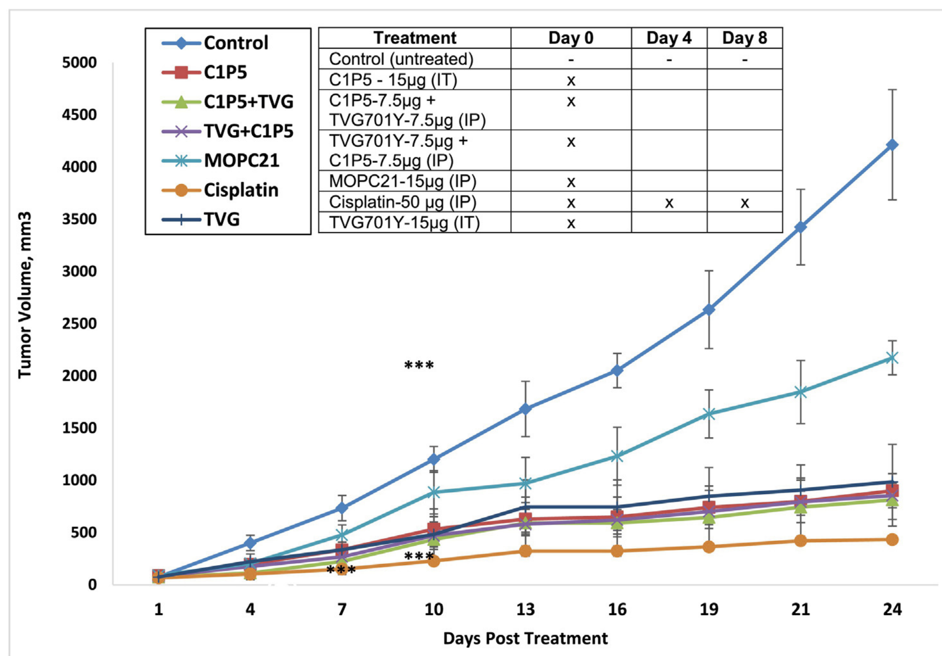
Figure 4. Increased necrosis correlates with additive doses of C1P5. H&E staining of CasKi tumors removed at the end of the 49th day of study period. Representative sections of: A) Untreated tumors appearance solid nests of tumor cells with intervening stroma B) Three IP injections of 50 μg Cisplatin- nest of tumor cells with minimal necrosis noted C) One IP injection of 15 μg C1P5 exhibited 30% necrosis D) Two IP injections of 15 μg C1P5 exhibited 60% necrosis E) Three IP injections of 15 μg C1P5 — 85% necrosis. Dose dependent tumor necrosis was observed in tumors treated with C1P5 with each additional dose given. Original magnification 20×.

approved usages of this approach in clinical practice [28]. Success depends on targeting HPV in such a way targets proteins involved in the development and maintenance of the disease. Several have the utilized the approach of hr-HPV antigen-specific immunotherapy to control metastases without damaging normal cells.

While the approaches of peptide-based vaccinations that involve the direct administration of peptides derived from HPV antigens have been explored, the low immunogenicity produced limits the efficacy in potency [29]. Alternative administration strategies such as intranasal route of administration, linkage of peptides to lipids or strategies to prevent peptide degradation, however much work needs to be added to enhance this therapeutic approach [30–32]. Gene therapy clinical trials evaluated the safety tolerability and immunogenicity of HPV E6 and E7 oncogenes in combination with immunotherapy and chemotherapy drugs are continually recruiting patients.

In our laboratory, we use mAbs against HPV E6 and E7 oncoproteins as a viral-specific approach for women with recurrent cervical cancer for future Phase 1 development [15,16]. We compared two different doses of C1P5 3ug/ml and 15ug/ml and found the single dose of either concentration was effective to control tumor. To extend the efficacy of treatment, we compared serial doses of C1P5 with Cisplatin and found tumor growth was inhibited in all treatment groups and a single dose of C1P5 was as effective as Cisplatin. We demonstrated that C1P5 and TVG701Y administration-related significant retardation of the tumor growth in mAb-treated groups was due to the mAbs specificity to HPV as irrelevant isotype control mAb MOPC21 was able to cause tumor growth inhibition initially, however the observed effect was lost after day 12 of observation.

There is recent clinical and pre-clinical data on using immunoglobulins for cancer therapy. The increasing number of pathologies where intravenous immunoglobulins (IVIg) display a beneficial



Treatment	Day 0	Day 4	Day 8
Control (untreated)	-	-	-
C1P5 - 15μg (IT)	x		
C1P5-7.5μg + TVG701Y-7.5μg (IP)	x		
TVG701Y-7.5μg + C1P5-7.5μg (IP)	x		
MOPC21-15μg (IP)	x		
Cisplatin-50 μg (IP)	x	x	x
TVG701Y-15μg (IT)	x		

Figure 5. Differential Treatment Strategies of Experimental CaSki Tumors. Mean tumor volume graph of CasKi tumors bearing mice showing suppressed tumor growth after intratumoral (IT) and intraperitoneal (IP) injections compared to untreated control. Tumor volume measured every three days during the 24 day observation period. Comparison to untreated control tumors and MOPC21 both demonstrate that anti-HPV E6 and E7 strategies inhibit tumor growth in a statistically sustained during days 16 to 24 compared to MOPC21.

action illustrates their therapeutic relevance [33,34]. Possible mechanisms of action include the ability of IVIg to modulate macrophage polarization toward M1-like phenotype, characterized by pro-inflammatory activity and inhibition of proliferation of cancer cells [35]. As our results point out, the E6 and E7 targeting antibodies cause complement deposition that appears to be triggered by the antibodies concentrations below the ones used in our work. The overall effect on the tumor reflects the contribution of specific complement deposition and non-specific immunoglobulin effect, probably, via macrophage polarization.

Here we show that C1P5 and TVG701Y monoclonal antibody treatment can significantly inhibit the tumor progression. Although combination treatment of C1P5 and TVG701Y was equally effective as independent C1P5 and TVG701Y treatment, combinatorial dosing regimens or intratumoral injections did not enhance therapeutic efficacy. These studies outlined in this report, collectively demonstrate that the anti-viral and anti-tumor properties that result in significant tumor growth inhibition underscore the definite potential of the translation capacity of this approach in a much-needed clinical setting.

Conclusion

Our long-term goal is to treat women with advanced, metastatic or recurrent cervical cancer in a Phase 1 clinical trial utilizing mAbs against HPV E6 and E7 oncoproteins. C1P5 and TVG701Y successfully inhibited the cervical tumor progression, have HPV-specific treatment and activation of immune system. While further refinement of our understanding of the anti-viral interactions are warranted, this approach is highly novel and promises low toxicity profiles which is of critical importance.

Acknowledgements

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