Published in final edited form as:

Vaccine. 2009 January 29; 27(5): 684–689. doi:10.1016/j.vaccine.2008.11.042.

Enhancing therapeutic HPV DNA vaccine potency through depletion of CD4⁺CD25⁺ T regulatory cells

Chi-Mu Chuang^{1,4,5}, Talia Hoory¹, Archana Monie¹, Annie Wu¹, Mei-Cheng Wang³, and Chien-Fu Hung^{1,2}

¹Department of Pathology, Johns Hopkins Medical Institutions, Baltimore, Maryland, USA

²Department of Oncology, Johns Hopkins Medical Institutions, Baltimore, Maryland, USA

³Department of Biostatistics, Johns Hopkins Medical Institutions, Baltimore, Maryland, USA

⁴Department of Obstetrics and Gynecology, Taipei Veterans General Hospital, Taipei, Taiwan

⁵School of Medicine, National Yang-Ming University, Taipei, Taiwan

Abstract

Therapeutic human papillomavirus (HPV) vaccines targeting E6 and/or E7 antigens represent an opportunity to control HPV-associated lesions. We have previously generated several therapeutic DNA vaccines targeting HPV-16 E7 antigen and generated significant antitumor effects. Since regulatory T cells (Tregs) play an important role in suppressing immune responses against tumors by immunotherapy, such as DNA vaccines, we tested if the therapeutic effects of a DNA vaccine encoding E7 linked to heat shock protein 70 (Hsp70) can be improved by a strategy to deplete Tregs using an anti-CD25 monoclonal antibody (PC61) in vaccinated mice. We found that administration of PC61 prior to vaccination with E7/Hsp70 DNA was capable of generating higher levels of E7-specific CD8⁺ T cells compared to the control antibody, leading to significantly improved therapeutic and long-term protective antitumor effects against an E7-expressing tumor, TC-1. Thus, a strategy to deplete CD4⁺CD25⁺ Tregs in conjunction with therapeutic tumor antigen-specific DNA vaccine may represent a potentially promising approach to control tumor. The clinical implications of our study are discussed.

Keywords

PC61; regulatory T cells (Tregs); DNA vaccine; heat shock protein 70 (Hsp70)

2.0 Introduction

Cervical cancer is the second leading cause of death by cancer for women worldwide. Close to half a million women are diagnosed each year with cervical cancer and about 2/5ths of these women do not survive [1]. The primary treatment for cervical cancer is radical hysterectomy and surgical debulking in combination with chemotherapy or radiotherapy. Patients with advanced cervical cancer still have a poor prognosis despite undergoing these conventional

Address correspondence to: Dr. Chien-Fu Hung, Department of Pathology, School of Medicine, Johns Hopkins University, CRB II Room 307, 1550 Orleans Street, Baltimore, Maryland 21231, USA. Phone: (410) 614-4906; Fax: (443) 287-4295; E-mail: chung2@jhmi.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

therapies with significant side effects. Thus, the development of innovative approaches to treatment remains a priority.

It is now clear that almost all cervical cancers are associated with human papillomavirus (HPV), particularly types 16 and 18. Two HPV oncogenic proteins, E6 and E7, are constantly expressed in cervical cancer and are responsible for the malignant transformation of cervical cancer. Thus, immunotherapy and vaccine development targeting E6 and/or E7 represents a great opportunity to control HPV-associated cervical malignancies. Numerous therapeutic vaccines targeting E6 and/or E7 have been reported in preclinical studies as well as early phases of clinical trials (for review, see [2]).

Antigen-specific immunotherapy using DNA vaccines has emerged as a promising alternative to current therapies for cervical cancer. Naked DNA vaccines are safe, very stable relative to protein vaccines and easy to reproduce (for review, see [3,4]). DNA vaccines are capable of generating tumor antigen-specific immune responses, which may potentially destroy tumors while sparing normal tissues. However, DNA vaccines are limited by a lack of immunogenicity and require additional means to improve their ability to generate strong immune responses. Using a gene gun administration system, it is now possible to directly deliver DNA vaccines to Langerhans cells in the skin, which are immature dendritic cells (DCs). Langerhans cells, once they uptake and express the DNA, will mature and migrate to the draining lymph nodes, where they prime and activate naïve cytotoxic T cells specific for tumor antigen (for review, see [5,6]). We have used this system to develop a number of strategies to enhance DNA vaccine potency by modifying the properties of DCs (for review, see [7,8]).

DNA vaccination employing intracellular targeting strategies to improve MHC class I and II presentation of the encoded antigen in DCs represents a potentially effective strategy to improve DNA vaccine potency. This strategy involves linking the tumor antigen to a chaperone protein, which includes members of the heat shock protein (Hsp) family. Hsps bound with antigens are concentrated in DCs and are able to activate other immature DCs [9-11]. We have previously shown that DNA vaccines encoding Hsp70 linked to model antigen, human papillomavirus type 16 (HPV-16) E7 antigen (E7/Hsp70 DNA) significantly increases the expansion and activation of E7-specific CD8⁺ T cells, resulting in enhanced antitumor immunity against established tumors [12].

The employment of anti-CD25 monoclonal antibody (PC61) represents another opportunity to enhance the potency of DNA vaccines because it targets naturally occurring CD4⁺CD25⁺ T cells. It has been shown that the administration of PC61 can lead to the depletion of CD4⁺CD25⁺ T cells [13]. T regulatory cells (Tregs) not only influence self-antigen specific immune responses, but also dampen foreign-antigen specific immunity. It has also been suggested that tumor cells secrete cytokines to attract Tregs to the tumor microenvironment (for review, see [14]). PC61 has been previously used in combination with a tumor cell-based vaccine, and has been shown to enhance the efficacy of the vaccine in treating melanoma tumors [15]. A comparable strategy has been used by Furuichi *et al* [16] and Toka *et al* [17] using murine viral infection models. In respect to anti-tumour immunity, studies have demonstrated significant improvement in CTL responses following T reg cell depletion [18]. Thus, Treg depletion using PC61 represents a potentially promising strategy to improve the potency of DNA vaccines.

In the current study, we investigate whether the depletion of CD4⁺CD25⁺ Tregs using PC61 would lead to the enhancement of HPV DNA vaccine potency in vaccinated mice. We found that administration of PC61 prior to vaccination with E7/Hsp70 DNA was capable of generating higher levels of E7-specific CD8⁺ T cells compared to administration of the isotype control antibody, leading to significantly improved therapeutic and long-term protective

antitumor effects against TC-1 tumors. Thus, a strategy to deplete CD4⁺CD25⁺ Tregs in conjunction with therapeutic tumor antigen-specific DNA vaccine may represent a potentially promising approach to control tumor. The clinical implications of our study are discussed.

3.0 Materials and Methods

3.1 Animals and tumor cell line

Female C57BL/6 mice, 5 to 6 week-old, were purchased from National Cancer Institute (Frederick, MD). Animals were used in compliance with institutional animal health care regulations, and all experimental procedures were approved by the Johns Hopkins Institutional Animal Care and Use Committee.

The production and maintenance of TC-1 cells has been described previously [19]. In brief, the cells were grown in RPMI 1640, supplemented with 10% (v/v) fetal bovine serum, 50 U/ml penicillin/streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate, 2 mM nonessential amino acids, and 0.4 mg/ml G418 at 37°C with 5% CO₂. On the day of tumor challenge, TC-1 cells were harvested by trypsinization, washed twice with 1X Hanks buffered salt solution, and finally resuspended in 1X Hanks buffered salt solution to the designated concentration for injection.

3.2 Antibodies, peptides and plasmid DNA Constructs

Antibodies against mouse CD4 (PE-conjugated, clone L3T4), CD8a (PE-conjugated, clone 53.6.7), IFN- γ (FITC-conjugated, clone XMG1.2), CD25 to check depletion (PerCP Cy5.5-conjugated, clone 7D4) and rat isotype-matched immunoglobulin were purchased from BD Pharmingen (San Diego, CA). Antibodies against CD25 for depletion (FITC-conjugated, clone PC61.5) were purchased from American Type Culture Collection (Manassas, VA). Rat anti-mouse CD25 mAbs (PC61) were used as ascites produced by PC61 hybridoma in *nu/nu* mice and purified by Prosep G Ig purification kit (Millipore, Bedford, MA).

The HPV-16 E7 peptide (aa49-57) was synthesized by Macromolecular Resources (Denver, CO) at a purity of $\geq 70\%$. The generation of pcDNA3-E7/Hsp70 plasmid construct has been described previously [12].

3.3 Depletion of CD4⁺CD25⁺ regulatory T cells with PC61

Two doses of monoclonal antibody PC61 were administered to C57BL/6 mice groups, 500 μ g or 170 μ g / mouse via intraperitoneal injection. As a negative control, rat isotype-matched antibody (isotype control Ab) was administered to C57BL/6 mice via intraperitoneal injection, using a dose of 500 μ g / mouse. Splenocytes were harvested and the cell surfaces were stained using anti-CD4 and anti-CD25 to determine levels of depletion. Persistence of CD4⁺CD25⁺ Treg depletion using 170 μ g / mouse of PC61 was then determined using time points day 4 and 14 after antibody injection. Groups of C57BL/6 mice were injected with 170 μ g / mouse of PC61 or isotype Ab as a control. Splenocytes were harvested and stained using anti-CD4 and anti-CD25 to determine levels of depletion at each time point.

3.4 DNA Vaccination following Treg Depletion

For the vaccination, DNA-coated gold particles were prepared according to a protocol described previously [12]. Gold particles coated with the pcDNA3-E7/Hsp70 DNA vaccine were delivered to the shaved abdominal regions of mice using a helium-driven gene gun (Bio-Rad, Hercules, CA) with a discharge pressure of 400 lb/in². Mice were then immunized with 2 μ g / mouse of the DNA vaccine twice with a 1-wk interval.

In the experiments using PC61 together with DNA vaccination, C57BL/6 mice were injected with 170 μg / mouse PC61 4 days prior to DNA vaccination with E7/Hsp70 DNA vaccine. As a negative control, a group of mice were injected with 170 μg of isotype control Ab.

3.5 Intracellular Staining and Flow Cytometry

To check for CD4⁺CD25⁺ T cells in the depletion experiments, splenocytes (3×10^5 /mouse) harvested from the mice were stained using anti-CD4 followed by anti-CD25 clone 7D4. After staining, flow cytometry analysis was performed using FACScalibur with CellQuest software (BD Biosciences, Mountain View, Calif.). To check for E7-specific CD8⁺ T cells, harvested splenocytes (3×10^5 /mouse) were incubated with 2 $\mu\text{g}/\text{ml}$ of E7 peptide (aa 49–57) containing an H-2 D^b-restricted CTL epitope to detect E7-specific CD8⁺ T-cell precursor responses. Golgistop (PharMingen, San Diego, CA) was added 6 h before harvesting the cells from the culture. Cells were subjected to intracellular cytokine staining by using the Cytofix/Cytoperm kit according to the manufacturer's instructions (BD Pharmingen). Intracellular gamma interferon (IFN- γ) was stained with fluorescein isothiocyanate-conjugated rat anti-mouse IFN- γ . Intracellular CD8a was stained with phycoerythrin-conjugated monoclonal rat anti-mouse CD8a.

3.6 *In Vivo* Long-Term Tumor Protection Experiments

C57BL/6 mice (5 per group) were administered 170 μg / mouse of PC61 or isotype control Ab 4 days prior to vaccination twice with a 1-wk interval with E7/Hsp70 DNA. One control group of mice was administered 170 μg / mouse of PC61 alone. A group of naïve mice used was another control. 53 days after the last vaccination, the mice groups were subjected to tumor challenge with 5×10^4 /mouse of TC-1 tumor cells in the right flank. Tumor sizes in the mice were monitored and measured every 3-4 days using digital calipers.

3.7 *In Vivo* Tumor Treatment Experiments

C57BL/6 mice (5 per group) were subjected to tumor inoculation using 5×10^4 TC-1 tumor cells/mouse in the right flank. Five days after tumor inoculation, groups of tumor-bearing mice were administered 170 μg / mouse of PC61 via intraperitoneal injection 4 days prior to vaccination with E7/Hsp70 DNA twice with a 1-wk interval. One group of tumor-bearing mice was administered PC61 alone as a control. A different group of tumor-bearing mice was left without treatment as another control. Tumor sizes in the mice were monitored and measured every 3-4 days using digital calipers.

3.8 Statistical Analysis

All data expressed as means $\pm$ standard deviation (SD) are representative of at least two different experiments. Comparisons between individual data points for tumor sizes were made using a Student's *t*-test or repeated measure ANOVA (analysis of variance) test. Differences in survival between experimental groups were analyzed using the Log rank test. Tumor sizes were calculated using the following equation: (tumor length $\times$ width $\times$ height) / 2. Death of mouse was arbitrarily defined as tumor diameter greater than 2 cm.

4.0 Results

4.1 Administration of anti-CD25 monoclonal antibody (PC61) leads to depletion of CD4⁺CD25⁺ T cells in PC61-treated mice

In order to determine whether treatment with anti-CD25 monoclonal antibody (PC61) would deplete mice of CD4⁺CD25⁺ T cells, we performed *in vivo* experiments using C57BL/6 mice. We administered doses of either 170 μg or 500 μg /mouse of PC61 via intraperitoneal injection based on previously determined pilot experiments. We also performed a negative control using

500 µg/mouse of rat isotype-control antibody (isotype Ab). Four days after the injection, splenocytes were harvested and checked for CD4⁺CD25⁺ T cells using flow cytometry analysis. As shown in Figure 1A, the percentages of CD4⁺CD25⁺ T cells from splenocytes were similar in the mice treated with both the doses of PC61. In comparison, the percentage of CD4⁺CD25⁺ T cells from splenocytes in mice treated with isotype Ab was significantly higher. We also compared the depletion in mice treated with 170 µg/mouse of PC61 at time points 4 and 14 days using isotype Ab as a negative control. As shown in Figure 1B, the percentages of CD4⁺CD25⁺ T regulatory cells from splenocytes in mice treated with 170 µg/mouse of PC61 were similar on day 4 and 14. Furthermore, the same trend was observed in the negative control. Taken together, our data suggests that the PC61 antibody at the dose of 170 µg/mouse leads to effective depletion of CD4⁺CD25⁺ T cells.

4.2 Depletion of CD4⁺CD25⁺ T regulatory cells with PC61 enhances the E7-specific CD8⁺ T cell immune responses generated by vaccination with E7/Hsp70 DNA

It has been shown that CD4⁺CD25⁺ T regulatory cells suppress the activation of effector T cells *in vitro* [20]. In order to determine if depletion of these CD4⁺CD25⁺ T regulatory cells in C57BL/6 mice would enhance the activation of E7-specific CD8⁺ T cells induced by vaccination with pcDNA3-E7/Hsp70 DNA, C57BL/6 mice (5 per group) were administered 170µg/mouse of PC61 or isotype control Ab via intraperitoneal injection and 4 days later, vaccinated with E7/Hsp70 DNA intradermally via gene gun twice with 1-week interval as depicted in Figure 2A. A group of naïve mice vaccinated with E7/Hsp70 DNA without antibody treated were used as negative controls. 1 week after the last vaccination, splenocytes were harvested from the different groups and characterized for E7-specific CD8⁺ T cells using intracellular cytokine staining followed by flow cytometry analysis. As shown in Figure 2B and C, mice treated with PC61 and E7/Hsp70 DNA demonstrated significantly greater E7-specific CD8⁺ T cell immune responses compared to the mice treated with isotype Ab and E7/Hsp70 DNA or E7/Hsp70 DNA only. Thus, our results indicate that depletion of CD4⁺CD25⁺ T regulatory cells using PC61 enhances the E7-specific CD8⁺ T cell immune responses generated by vaccination with E7/Hsp70 DNA in treated mice.

4.3 PC61 enhances the long-term protective antitumor effects generated by vaccination with E7/Hsp70 DNA

To determine if the observed enhancement of E7-specific CD8⁺ T cell immune responses generated by vaccination with E7/Hsp70 DNA by PC61 would translate into long-term tumor protection, we performed *in vivo* tumor protection experiments using C57BL/6 mice. C57BL/6 mice (5 per group) were administered 170µg/mouse of PC61 or isotype control Ab via intraperitoneal injection 4 days later, vaccinated with E7/Hsp70 DNA intradermally via gene gun twice with 1-week interval as depicted in Figure 2A. One group of mice was administered PC61 alone and another group of untreated mice was used as a negative control. All groups of mice were challenged with TC-1 tumor cells 53 days after the last vaccination. We observed that all the TC-1 tumor bearing mice showed increased numbers of T regulatory cells in the lymphoid tissues and in tumor infiltrating lymphocytes compared to naïve mice [21, 22]. Furthermore, we also found that PC61 injection can result in the depletion of T regulatory cells without significantly changing the composition of CD4 and CD8 effector cells in the tumor-infiltrating lymphocytes (data not shown). As shown in Figure 3A, the mice treated with PC61 and vaccinated with E7/Hsp70 DNA before tumor challenge exhibited significantly decreased tumor growth compared to vaccinated mice treated with the isotype control Ab (* *p*=0.017). We further characterized the survival in the different mice groups using Kaplan-Meier survival analysis. As shown in Figure 3B, the mice treated with PC61 and vaccinated with E7/Hsp70 DNA before tumor challenge exhibited significantly improved survival compared to vaccinated mice treated with the isotype control Ab (** *p*=0.039). Taken together, our data indicate that depletion of CD4⁺CD25⁺ T regulatory cells with PC61 can enhance the long-term

protective antitumor effects generated by vaccination with E7/Hsp70 DNA and improve the survival in tumor-challenged mice.

4.4 PC61 enhances the therapeutic antitumor effects generated by treatment of TC-1 tumor-bearing mice with E7/Hsp70 DNA

In order to demonstrate whether PC61 can enhance the antitumor effects generated by treatment of tumor-bearing mice with E7/Hsp70 DNA vaccine, we performed *in vivo* tumor treatment experiments. C57BL/6 mice were challenged with 5×10^4 /mouse of TC-1 tumor cells on day 0. Five days after tumor challenge, mice were administered 170 μ g/mouse of PC61 or isotype control Ab via intraperitoneal injection then were 4 days later, vaccinated with E7/Hsp70 DNA intradermally via gene gun twice with 1-week interval as depicted in Figure 2A. One group of mice was administered PC61 alone and another group of untreated mice was used as a negative control. As shown in Figure 4A, the tumor-bearing mice treated with PC61 followed by E7/Hsp70 DNA vaccine exhibited significantly decreased tumor size compared to the tumor-bearing mice treated with the isotype control Ab followed by E7/Hsp70 DNA vaccine (* $p=0.127$). We further characterized the survival of the different mice groups using Kaplan-Meier survival analysis. As shown in Figure 4B, the tumor-bearing mice treated with PC61 followed by E7/Hsp70 DNA exhibited significantly improved survival compared to vaccinated mice treated with the isotype control Ab (** $p=0.094$). Taken together, our data indicate that PC61 enhances the therapeutic antitumor effects generated by treatment with E7/Hsp70 DNA and prolongs survival in tumor-bearing mice.

5.0 Discussion

In the current study, we demonstrate the efficient depletion of CD4⁺CD25⁺ T regulatory cells using the anti-CD25 monoclonal antibody PC61. Furthermore, we showed that administration of PC61 prior to vaccination with E7/Hsp70 DNA generated significantly higher levels of E7-specific CD8⁺ T cells compared to administration of isotype Ab with DNA vaccination. In addition, treatment with PC61 before DNA vaccination led to significantly greater long-term protective and therapeutic antitumor effects against TC-1 tumor challenge compared to vaccination alone. Thus, combining a strategy to reduce the levels of Tregs utilizing PC61 with an intracellular targeting strategy utilizing E7/Hsp70 DNA vaccine represents an effective approach to improve the potency of therapeutic HPV DNA vaccines.

Increasing evidence suggests that it is important to modify the tumor microenvironment in order to improve effectiveness of immunotherapy (for review see [23]). Studies have shown that tumors secrete the chemokine CCL22, which is chemotactic for CD4⁺ CD25⁺ T regulatory cells, which may recruit the Tregs to the tumor microenvironment to suppress cytotoxic activity of effector T cells [24,25]. Furthermore, it has been recently suggested that immunosuppression via CD4⁺CD25⁺ T regulatory cell recruitment is one of the major evasion mechanisms of tumors (for review, see [14]) and a key obstacle to successful cancer immunotherapy [26-28]. Therefore, curtailing the influence of immune suppressor cells such as CD4⁺CD25⁺ Tregs is an important approach to improving the potency of HPV DNA vaccines.

An important limitation in the depletion of Tregs using PC61 Ab is lack of specificity for functionally suppressive CD4⁺ T cells. Activated CD4⁺ T cells have also been shown to express CD25, however the level of CD25 is significantly lower in these cells (for reviews on CD4⁺CD25⁺ T cells, see [27,29]). In addition to using CD25 to identify CD4⁺CD25⁺ T cells, it may be of interest to utilize other markers for these cells. Nuclear transcription factor forkhead box P3 (FOXP3) has been heavily associated with Tregs since it is required for the development of natural Tregs [30-32] and in many cases has been used for the identification for Tregs [33]. However, it has been found that not all CD4⁺CD25⁺FOXP3⁺ T cells have regulatory function and some FOXP3 negative T cells are also suppressive in nature [30,34].

It has been recently proposed that low levels of phosphodiesterase 3B can be used to identify natural CD4⁺CD25⁺ Tregs [34]. Thus, further investigation into functional markers for CD4⁺CD25⁺ T regulatory cells will allow us to improve methods for specific targeting of CD4⁺CD25⁺ T regulatory cells, which may further enhance the effectiveness of immunotherapeutic strategies.

In summary, combining a strategy to deplete Tregs with PC61 Ab with a therapeutic HPV DNA vaccine represents a potentially promising approach for the control of HPV-associated lesions. Our strategies to deplete T regulatory cells include the employment of cimetidine [35], sunitinib [36], cyclophosphamide [37] and several other chemotherapeutic agents. For example, cimetidine has been recently shown to enhance immune response of DNA vaccination by impairing the regulatory function of Tregs [35]. Thus, it will be important to identify the best approach for specific depletion of T regulatory cells for future clinical translation.

Acknowledgements

We would like to thank Dr. T.-C. Wu for helpful discussions and critical review of the manuscript. This work was supported by 1 RO1 CA114425 01, the Cervical Cancer SPORE Program (P50 CA098252) of the NCI and the American Cancer Society (ACS).

References

1. Parkin DM, Bray F, Ferlay J, Pisani P. Global cancer statistics, 2002. *CA Cancer J Clin* 2005 Mar-Apr;55(2):74–108. [PubMed: 15761078]
2. Hung CF, Monie A, Alvarez RD, Wu TC. DNA vaccines for cervical cancer: from bench to bedside. *Exp Mol Med* 2007 Dec 31;39(6):679–89. [PubMed: 18160838]
3. Donnelly JJ, Ulmer JB, Shiver JW, Liu MA. DNA vaccines. *Annu Rev Immunol* 1997;15:617–48. [PubMed: 9143702]
4. Gurunathan S, Klinman DM, Seder RA. DNA vaccines: immunology, application, and optimization*. *Annu Rev Immunol* 2000;18:927–74. [PubMed: 10837079]
5. Condon C, Watkins SC, Celluzzi CM, Thompson K, Falo LD Jr. DNA-based immunization by in vivo transfection of dendritic cells. *Nat Med* 1996 Oct;2(10):1122–8. [PubMed: 8837611]
6. Porgador A, Irvine KR, Iwasaki A, Barber BH, Restifo NP, Germain RN. Predominant role for directly transfected dendritic cells in antigen presentation to CD8⁺ T cells after gene gun immunization. *J Exp Med* 1998 Sep 21;188(6):1075–82. [PubMed: 9743526]
7. Hung CF, Yang M, Wu TC. Modifying professional antigen-presenting cells to enhance DNA vaccine potency. *Methods Mol Med* 2006;127:199–220. [PubMed: 16988456]
8. Tsen SW, Paik AH, Hung CF, Wu TC. Enhancing DNA vaccine potency by modifying the properties of antigen-presenting cells. *Expert Rev Vaccines* 2007 Apr;6(2):227–39. [PubMed: 17408372]
9. Singh-Jasuja H, Toes RE, Spee P, Munz C, Hilf N, Schoenberger SP, et al. Cross-presentation of glycoprotein 96-associated antigens on major histocompatibility complex class I molecules requires receptor-mediated endocytosis. *J Exp Med* 2000 Jun 5;191(11):1965–74. [PubMed: 10839811]
10. Spee P, Neefjes J. TAP-translocated peptides specifically bind proteins in the endoplasmic reticulum, including gp96, protein disulfide isomerase and calreticulin. *Eur J Immunol* 1997 Sep;27(9):2441–9. [PubMed: 9341791]
11. Srivastava PK, Udono H, Blachere NE, Li Z. Heat shock proteins transfer peptides during antigen processing and CTL priming. *Immunogenetics* 1994;39(2):93–8. [PubMed: 8276462]
12. Chen CH, Wang TL, Hung CF, Yang Y, Young RA, Pardoll DM, et al. Enhancement of DNA vaccine potency by linkage of antigen gene to an HSP70 gene. *Cancer Res* 2000 Feb 15;60(4):1035–42. [PubMed: 10706121]
13. Yu P, Lee Y, Liu W, Krausz T, Chong A, Schreiber H, et al. Intratumor depletion of CD4⁺ cells unmasks tumor immunogenicity leading to the rejection of late-stage tumors. *J Exp Med* 2005 Mar 7;201(5):779–91. [PubMed: 15753211]

14. Curiel TJ. Tregs and rethinking cancer immunotherapy. *J Clin Invest* 2007 May;117(5):1167–74. [PubMed: 17476346]
15. Suttmuller RP, van Duivenvoorde LM, van Elsas A, Schumacher TN, Wildenberg ME, Allison JP, et al. Synergism of cytotoxic T lymphocyte-associated antigen 4 blockade and depletion of CD25(+) regulatory T cells in antitumor therapy reveals alternative pathways for suppression of autoreactive cytotoxic T lymphocyte responses. *J Exp Med* 2001 Sep 17;194(6):823–32. [PubMed: 11560997]
16. Furuichi Y, Tokuyama H, Ueha S, Kurachi M, Moriyasu F, Kakimi K. Depletion of CD25+CD4+T cells (Tregs) enhances the HBV-specific CD8+ T cell response primed by DNA immunization. *World J Gastroenterol* 2005 Jun 28;11(24):3772–7. [PubMed: 15968737]
17. Toka FN, Suvas S, Rouse BT. CD4+ CD25+ T cells regulate vaccine-generated primary and memory CD8+ T-cell responses against herpes simplex virus type 1. *J Virol* 2004 Dec;78(23):13082–9. [PubMed: 15542660]
18. Van Meirvenne S, Dullaers M, Heirman C, Straetman L, Michiels A, Thielemans K. In vivo depletion of CD4+CD25+ regulatory T cells enhances the antigen-specific primary and memory CTL response elicited by mature mRNA-electroporated dendritic cells. *Mol Ther* 2005 Nov;12(5):922–32. [PubMed: 16257383]
19. Lin KY, Guarnieri FG, Staveley-O'Carroll KF, Levitsky HI, August JT, Pardoll DM, et al. Treatment of established tumors with a novel vaccine that enhances major histocompatibility class II presentation of tumor antigen. *Cancer Res* 1996 Jan 1;56(1):21–6. [PubMed: 8548765]
20. Thornton AM, Shevach EM. Suppressor effector function of CD4+CD25+ immunoregulatory T cells is antigen nonspecific. *J Immunol* 2000 Jan 1;164(1):183–90. [PubMed: 10605010]
21. Peng S, Trimble C, Alvarez RD, Huh WK, Lin Z, Monie A, et al. Cluster intradermal DNA vaccination rapidly induces E7-specific CD8+ T-cell immune responses leading to therapeutic antitumor effects. *Gene Ther* 2008 Aug;15(16):1156–66. [PubMed: 18401437]
22. Tseng CW, Hung CF, Alvarez RD, Trimble C, Huh WK, Kim D, et al. Pretreatment with cisplatin enhances E7-specific CD8+ T-Cell-mediated antitumor immunity induced by DNA vaccination. *Clin Cancer Res* 2008 May 15;14(10):3185–92. [PubMed: 18483387]
23. Drake CG, Jaffee E, Pardoll DM. Mechanisms of immune evasion by tumors. *Adv Immunol* 2006;90:51–81. [PubMed: 16730261]
24. Curiel TJ, Coukos G, Zou L, Alvarez X, Cheng P, Mottram P, et al. Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. *Nat Med* 2004 Sep;10(9):942–9. [PubMed: 15322536]
25. Iellem A, Mariani M, Lang R, Recalde H, Panina-Bordignon P, Sinigaglia F, et al. Unique chemotactic response profile and specific expression of chemokine receptors CCR4 and CCR8 by CD4(+)CD25(+) regulatory T cells. *J Exp Med* 2001 Sep 17;194(6):847–53. [PubMed: 11560999]
26. Dunn GP, Old LJ, Schreiber RD. The immunobiology of cancer immunosurveillance and immunoediting. *Immunity* 2004 Aug;21(2):137–48. [PubMed: 15308095]
27. Shevach EM. CD4+ CD25+ suppressor T cells: more questions than answers. *Nat Rev Immunol* 2002 Jun;2(6):389–400. [PubMed: 12093005]
28. Zou W. Immunosuppressive networks in the tumour environment and their therapeutic relevance. *Nat Rev Cancer* 2005 Apr;5(4):263–74. [PubMed: 15776005]
29. Bach JF. Regulatory T cells under scrutiny. *Nat Rev Immunol* 2003 Mar;3(3):189–98. [PubMed: 12658267]
30. Curiel TJ. Regulatory T-cell development: is Foxp3 the decider? *Nat Med* Mar;200713(3):250–3.
31. Fontenot JD, Gavin MA, Rudensky AY. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. *Nat Immunol* 2003 Apr;4(4):330–6. [PubMed: 12612578]
32. Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. *Science* 2003 Feb 14;299(5609):1057–61. [PubMed: 12522256]
33. Graca L, Chen TC, Le Moine A, Cobbold SP, Howie D, Waldmann H. Dominant tolerance: activation thresholds for peripheral generation of regulatory T cells. *Trends Immunol* 2005 Mar;26(3):130–5. [PubMed: 15745854]
34. Gavin MA, Rasmussen JP, Fontenot JD, Vasta V, Manganiello VC, Beavo JA, et al. Foxp3-dependent programme of regulatory T-cell differentiation. *Nature* 2007 Feb 15;445(7129):771–5. [PubMed: 17220874]

35. Wang J, Su B, Ding Z, Du X, Wang B. Cimetidine enhances immune response of HBV DNA vaccination via impairment of the regulatory function of regulatory T cells. *Biochem Biophys Res Commun* 2008 Aug 1;372(3):491–6. [PubMed: 18502198]
36. Finke JH, Rini B, Ireland J, Rayman P, Richmond A, Golshayan A, et al. Sunitinib reverses type-1 immune suppression and decreases T-regulatory cells in renal cell carcinoma patients. *Clin Cancer Res* 2008 Oct 15;14(20):6674–82. [PubMed: 18927310]
37. Machiels JP, Reilly RT, Emens LA, Ercolini AM, Lei RY, Weintraub D, et al. Cyclophosphamide, doxorubicin, and paclitaxel enhance the antitumor immune response of granulocyte/macrophage-colony stimulating factor-secreting whole-cell vaccines in HER-2/neu tolerized mice. *Cancer Res* 2001 May 1;61(9):3689–97. [PubMed: 11325840]

Figure 1. Characterization of depletion of CD4⁺CD25⁺ T cells in mice treated with anti-CD25 antibody (PC61)

(A) Representative flow cytometry data demonstrating the percentages of CD4⁺CD25⁺ lymphocytes in the spleens of mice treated with different doses of the PC61 Ab. C57BL/6 mice (5 per group) were intraperitoneally administered anti-CD25 monoclonal antibody (PC61) in two doses, 170 µg or 500 µg/mouse. Another group of mice was intraperitoneally injected with 500 µg/mouse of rat isotype-matched antibody (isotype Ab) as a negative control. Splenocytes were harvested on day 4 after intraperitoneal injection of antibody and then subjected to flow cytometry analysis for CD4 and CD25 using the anti-CD4 and anti-CD25 antibodies. *Note:* The dose of 170 µg/mouse achieved a level of CD4⁺CD25⁺ T cell depletion close to the level of depletion using the dose of 500 µg. **(B)** Bar graph depicting the percentages of CD4⁺CD25⁺ T cells from the splenocytes of mice treated with PC61 or isotype Ab on D4 and D14 (mean ± SD). C57BL/6 mice (5 per group) were i.p. administered 170 µg/mouse of PC61 or isotype Ab (negative control). Splenocytes were harvested at day 4 and 14 after administration of antibody and subjected to flow cytometry analysis for CD4 and CD25 using the relevant antibodies. Data shown are representative of two experiments performed.

Figure 2. Characterization of E7-specific CD8⁺ T cell responses in mice administered PC61 followed by E7/HSP70 DNA

(A) Schematic diagram for the treatment regimen for mice receiving PC61, isotype Ab and/or E7/Hsp70 DNA vaccine. C57BL/6 mice (**5 per group**) were i.p. administered 170 μ g/mouse of PC61 or isotype Ig as a negative antibody control on day 0. Four days later, the mice were vaccinated with 2 μ g/mouse of E7/Hsp70 DNA intradermally via gene gun twice with a 1-week interval. A group of C57BL/6 mice vaccinated with E7/Hsp70 DNA without antibody treatment were used as controls. Ten days after the last vaccination, splenocytes were harvested, stimulated with E7 epitope (aa49-57) and characterized for E7-specific CD8⁺ T cells using intracellular cytokine staining followed by flow cytometry analysis. **(B)** Representative flow cytometry data depicting the **percentage** of E7-specific CD8⁺ IFN- γ ⁺ T cells in each of the treatment groups. **(C)** Bar graph depicting the ratio of E7-specific CD8⁺ IFN- γ ⁺ T cells in mice treated with PC61 and E7/Hsp70 DNA or isotype Ig and E7/Hsp70 DNA compared to the no-antibody control. The *p*-values for the groups with statistical significance are indicated. Data shown is the average of three independent experiments (mean $\pm$ SEM).

Figure 3. *In vivo* long-term tumor protection experiments

C57BL/6 mice (5 per group) were administered 170 μ g/mouse of PC61 or isotype Ab via intraperitoneal injection followed by E7/Hsp70 DNA vaccination 4 days later twice with a 1-week interval as depicted in Figure 2A. One group of naïve mice was administered PC61 alone and another group of untreated mice was used as a negative control. 53 days after the last vaccination, all mice groups were subcutaneously challenged with 5×10^4 TC-1 tumor cells/mouse by tail vein injection. Tumor sizes in the mice were monitored every 3-4 days using digital calipers. (A) Line graph depicting the tumor sizes over time of tumor-challenged mice in the different groups. (B) Kaplan-Meier survival curve of the different groups of tumor-challenged mice. * indicates $p = 0.017$; ** indicates $p = 0.039$ (mean $\pm$ SD).

Figure 4. *In vivo* tumor treatment experiments

C57BL/6 mice (5 per group) were inoculated with 5×10^4 /mouse of TC-1 tumor cells via subcutaneous tail vein injection. Five days after tumor inoculation, tumor-bearing mice were administered 170 μ g/mouse of PC61 or isotype control Ab followed by treatment 4 days later with 2 μ g/mouse of E7/Hsp70 DNA twice with a 1-week interval. One group of tumor-bearing mice was administered PC61 alone and another group of tumor-bearing mice was left untreated as a negative control. Tumor sizes were monitored every 3-4 days using digital calipers. **(A)** Line graph depicting the tumor sizes in tumor-bearing mice treated with the different regimens. **(B)** Kaplan-Meier survival curve of the mice treated with the various regimens. The *p*-values for the groups with statistical significance are indicated. * indicates *p* = 0.127; ** indicates *p* = 0.094 (mean $\pm$ SD).