# DEVELOPMENT OF A SIMPLE BUT EFFECTIVE CANCER VACCINE CONSISTING OF AN ANTIGEN AND A CATIONIC LIPID

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**DEVELOPMENT OF A SIMPLE BUT EFFECTIVE CANCER VACCINE CONSISTING OF AN ANTIGEN AND A CATIONIC LIPID**

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Human papillomavirus (HPV) oncoproteins E6 and E7 which are constitutively expressed in cervical cancer cells are ideal targets for developing immunotherapies for treatment of existing HPV-associated carcinoma. In this project, we developed a simple, safe, and efficient, peptide-based therapeutic cancer vaccine, DOTAP/E7 complex, which comprises only two molecules: a DOTAP cationic lipid and an MHC class I-restricted peptide antigen derived from HPV-16 E7 protein. TC-1 cell line which is HPV-16 $E7^+$ was used as a tumor model in an $H\text{-}2^b$ murine system. Tumor-bearing mice showed significant tumor inhibition following a single injection of DOTAP/E7 at the optimal lipid dose, suggesting that DOTAP liposome alone can be a potent adjuvant. E7 peptide formulated with DOTAP was taken up by dendritic cells (DC) and induced DC activation and migration to the draining lymph node (DLN), eliciting antigen-specific $CD8^+$ cytotoxic T lymphocyte (CTL) responses. The mechanism of DOTAP as a vaccine adjuvant was revealed by DOTAP-mediated reactive oxygen species (ROS) production in DC. At the optimal lipid dose, DOTAP/E7 generates an adequate level of ROS for the initiation of the vaccine mechanism. In addition, we have improved the vaccine formulation by incorporation of E7-lipopeptide instead of the water-soluble native E7 peptide. The improved DOTAP/E7-lipopeptide vaccine showed a significantly enhanced therapeutic effect, including CTL response and anti-tumor activity. The vaccine was also effective for suppression of tumor growth in later

stages of tumor progression, suggesting applications for advanced cancer treatment. Furthermore, we extended the studies of the vaccine efficacy observed in the mouse model to human cells *in vitro*. Instead of H-2D$^b$-restricted peptide, an HLA-A2-restricted E7 peptide epitope (hE7) was formulated into the liposome. *In vitro* stimulation of naïve HLA-A2$^+$ human T lymphocytes by DOTAP/hE7-activated autologous DC elicited a stronger clonal T cell proliferation and higher HPV-specific CTL response compared to those stimulated with DC pulsed with hE7 peptide alone. The *in vivo* CTL and anti-tumor activity induced by DOTAP/hE7 vaccine were demonstrated in an HLA-A2 transgenic mouse model. Overall, our data suggest that DOTAP/hE7 is a potent therapeutic cancer vaccine formulation with potential for clinical applications for the treatment of HPV-related neoplasia.

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**PREFACE**

My thanks to my beloved friends and families.

Special thanks to Shilling and Leaf.

Chapel Hill, October 2007

(Just married!)

# 1.0 INTRODUCTION

## 1.1 INDUCTION OF IMMUNE RESPONSES AGAINST CANCER

### 1.1.1 Tumor immunology: from immune surveillance to immune escape

Immune surveillance was first identified in the early 20$^{th}$ century by Ehrlich et al. as a phenomenon that recognizes the transformed cells and destroys them before they are clinically detected. A virus-infected cell or tumor cells can be caught and eliminated by NK cells in the innate immune system [1]. However, tumor cells display multiple mechanisms to avoid the recognition by the innate and adoptive immune responses [2]. Indeed, several therapeutic vaccine studies have failed to inhibit the tumor growth *in vivo* even if they were capable of generating tumor associated antigen (TAA) specific cytotoxicity. In order to develop a suitable immunotherapy for treatment of cancer, it is important to understand the strategies that tumor cells use to escape from the immune surveillance. A current concept of the tumor immunoediting which leads to immune escape was proposed. It consists of elimination, equilibrium and escape [3].

During the elimination stage, the nascent tumors are detected by innate immune response and some of the transformed cells are killed by IFN-γ secreted by NK/NKT cells [4]. Adaptive immunity is initiated by ingestion of dead tumor cells by dendritic cells (DC). Migration of

mature DC to the draining lymph node followed by antigen presentation to naïve $CD4^+$ or $CD8^+$ cells essentially generates tumor cell antigen-specific T lymphocytes [5]. If any tumor cell variants have survived the elimination process, those cells continue to grow under an equilibrium status in the presence of IFN-γ and other immune cells [6]. Under this condition, the new tumor variants (usually the mutant) become less immunogenic and resistant to immune attack. The phase of equilibrium could be the longest among the three stages [7]. Eventually, tumor cells escape from immune surveillance and expand in an immunologically intact condition. In order to keep tumor progression, tumor cells also adopt some immunosuppressive strategies as follows:

**Impairment of TCR signaling**: Tumor cells-induced CD3ζ chain decrease in tumor infiltrating T cells (effectors), impairs the recognition of antigen and TCR-CD3 complex. Several clinical studies confirmed defects from CTL in patients with different cancer types [8] [9].

**Secretion of immunosuppressive factors**: A variety of tumor-derived soluble proteins such as VEGF, TGF-β, IL-10 have been extensively studied in the recent decade [10] [11] [12]. These immunosuppressive cytokines are produced in the tumor microenvironment and inhibit T cell activation and proliferation.

**Activation of negative regulatory factors**: Tumor cells produce FasL or an immunoregulatory enzyme indoleamine 2,3-dioxygenase (IDO) which promotes apoptosis in effector cells [13]. In addition, cancer cells also induce the activation of CTLA-4 and programmed death 1 (PD-1) in T cells. These molecules bind to B7 molecules to block the costimulatory signaling [14] [15].

**Regulatory T cells (Treg)**: Significant interest has recently focused on $CD4^+CD25^+Foxp3^+$ Treg. Many Treg populations can be found in the lung, breast, ovary and

skin cancer patients [16]. Increased Treg population in tumor and malignant ascites inversely correlates with the low survival rate of cancer patient.

### 1.1.2 Dendritic cells activation and toll-like receptors

Dendritic cells (DC) are considered the most effective antigen-presenting cells (APC) for generating immune responses. Since presentation of antigens to the immune system by appropriate professional APC is essential to elicit a strong immune reaction, DC holds great promise to improve the efficacy of cancer vaccines. At least six subsets of DC have been described in mouse including myeloid DC (mDC), plasmacytoid DC (pDC) and Langerhans cells which are also found in human [17]. mDC are potent APC and are associated with T cell activation, while pDC are considered to be specialized to secret type I interferon after stimulation with a virus. Both lymphoid and myeloid progenitors in bone marrow are able to differentiate into pDC and mDC [18]. Antigens can be taken up by DC in the peripheral tissue via recognition of DC surface receptors such as scavenger receptor or toll-like receptors (TLR). And the signals mediated through TLR enable DC maturation and migration to the lymph node.

TLRs were identified as the major recognition receptor for pathogen associated molecule pattern (PAMP) such as LPS, peptidoglycan, lipoteichoic acid and CpG-containing oligonucleotides (CpG ODN) [19] [20] [21]. Accumulated evidence has demonstrated that TLRs are the critical link between the innate and the adaptive immunities. TLRs are membrane proteins containing leucine-rich repeats (LRR) and an intracellular IL-1R-like domain [22] [23]. More than twelve TLRs have been identified and different TLRs can exert distinct but overlapping sets of biological effects [24]. All of these molecules are involved in engaging and

controlling the innate immune response, which is essential for pathogen elimination, and in orchestrating the transition to an adaptive immune response [25].

The link between adjuvants and TLRs in triggering adaptive immunity as well as subsequent signaling pathways has been a subject of many recent investigations. An adjuvant alerts the host immune system through a mechanism similar to that of an infection by a pathogen, which may involve interaction with a TLR followed by a danger signal to the immune system. For example, the unmethylated bacterial CpG-DNA, recognized by TLR9, was used as an adjuvant for cancer therapy to induce a helper T cell 1 (Th1)-type T-cell response. TLR9-deficient mice did not show any responses to CpG-DNA, including Th1 cytokine production, B-cell proliferation, and maturation of DCs [19]. Subsequent signal transduction activated by TLRs involves two major pathways [26]. The first pathway includes the activation of transcription factor NF-κB, which acts as a master switch for inflammation, regulating the transcription of many genes that encode proteins involved in immunity and inflammation. The second pathway triggers the activation of p38 and Jun amino-terminal (JNK) MAP kinases. Although NF-κB and MAP kinases can be activated by all TLRs, there are differences in the ultimate gene expression profile induced by individual TLRs

### 1.1.3 Activation of cytotoxic T lymphocytes

Induction of cytotoxic T lymphocyte (CTL) response is essential for eliminating virus-infected cells and tumor cells. During adaptive immune responses, CTL activation requires at least two signals: antigen recognition and costimulatory interaction. Mature DC migrate to the secondary lymphoid tissue such as spleens, LN where they stimulate naïve T cells by presentation of antigen-major histocompatibility (MHC) complex (signal 1). Antigen presentation alone can

activate pathogen-specific T-cells, but is not sufficient to trigger efficient T-cell expansion. Clonal T-cell expansion requires an additional signal delivered by costimulatory molecules such as CD80 and CD86. Signal 2 involves the interaction between costimulatory molecules on DC and CD28 on T cells. In absence of signal 2, T cells recognizing the antigen presented by DC are tolerized [27]. Many recent studies have shown that $CD4^+$ helper T cells are required for efficiently priming of memory $CD8^+$ CTLs. The cross-talk between $CD4^+$ T cells and DC essentially results in IL-12 production by DC, leading to a Th1 immune response.

### 1.1.4 Chemokines

The chemokines are a superfamily of small secretory peptides which were originally identified as potent chemoattractants for inflammatory cells including neutrophils, eosinophils, and monocytes/macrophages [28]. Subsequent studies have revealed that chemokines, especially CCR7 and its ligands (CCL19 and CCL21) have profound effects on immune reactions by regulating the trafficking of DC as well as other lymphocytes into the secondary lymphoid organs [29]. Since lymphatic endothelial cells are known to constitutively produce CCL21, The migration of mature DC from peripheral tissue to DLN and the antigen presentation to T cells are reflected by CCR7 expression [30]. Eventually, a small population of central memory T cells ($CCR7^+CD62L^+$) will lose their LN-homing receptors and become effector memory T cells ($CCR7^-CD62L^+$). The effector memory T cells leave LN and play important roles in eliminating tumor cells with distinctive antigens.

MCP-1/CCL2, a member of CC chemokines, was originally identified as chemotactic and activating factor for monocytes/macrophages [31]. Subsequent studies showed that it can also affect the function of T-cells, natural killer cells and neutrophils [32]. Further studies found that

MCP-1/CCL2 was the most potent activator of $CD8^+$ CTL activity [33], in the presence of Th1 cytokines, IL-12 and IFN-γ. Recently, it has been shown that chemokines enhance immunity by guiding naïve $CD8^+$ T cells to sites of $CD4^+$ T cell-dendritic cell interaction and promote memory $CD8^+$ T-cell generation [34] [35].

## 1.2 DEVELOPMENT OF THE CERVICAL CANCER VACCINE

### 1.2.1 Human papillomavirus and cervical cancer

Cancer of the uterine cervix is the second most common cancer among women worldwide. The incidence rate is about 10 per 100,000 in developed countries and 40 per 100,000 in developing countries, with approximately 500,000 new cases being diagnosed each year [36]. Among these patients, 45% of them die usually as a result of distant metastases. It is widely accepted that the human papillomaviruses (HPV) are the cause of nearly all cervical cancers and over 99.7% of cervical cancers contain HPV DNA, typically of the HPV subtypes 16, 18 and 31. In addition to cancers, HPV infection can also cause genital warts [37] and recurrent respiratory papillomatosis (RRP) [38]. More than 100 HPV subtypes have been characterized and the most common type of HPV associated with cervical cancer is type 16, which causes more than 50% of all cases [39]. Over the past 20 years the link between cervical cancer and HPV has been extensively studied.

HPV belongs to the family papovaviridae and contains a circular double stranded DNA genome. Upon infection, HPV randomly integrates into the genome of host cells and induce malignant transformation. The HPV genome can be separated into three distinct regions: the early (E) genes, late (L) genes and the non-coding upstream regulatory region (URR) [40].

Proteins encoded in the E region of the viral genome such as the E6 and E7, are important for inducing and maintaining the transformed phenotype [41] [42]. L region encodes genes for capsid proteins. One of the most studied mechanisms of the E6 and E7 oncoproteins is their abilities to alternate the functions of tumor-suppressor genes, p53 and retinoblastoma (RB). The p53 recognizes the damaged DNA and arrests the cell cycle at G1 phase for DNA repairing. If the repair does not occur, p53 induces the apoptosis of the damaged cells. In cervical cancer cells, E6 oncoprotein silences the function of p53 by degradation through ubiquitin system [43]. Similarly, RB also regulates the cell cycle and E7 oncoprotein inactivate its function by binding to phosphorylated RB [44]. As a result of p53 silencing and RB blocking, HPV infected cell are allowed to grow unregulated.

Treatment options for cervical neoplasia depend on the stage of the disease. The non-invasive cervical intraepithelial neoplasia (CIN) is treated with cryotherapy or laser therapy that destroys and removes the abnormal tissue. For early invasive or advanced cancers, internal/external radiation therapy and chemotherapy are given to the patients [45].

### 1.2.2 Preventative vaccine against HPV viral infection

Since HPV is the etiological agent of cervical neoplasia, preventing infection with the virus will also prevent development of cervical cancer. Merck's HPV vaccine, GARDASIL™, which is a preventive vaccine against HPV infection, is already available in the market. This exciting development for an HPV preventive vaccine is based on immunization of individuals with HPV virus-like particles (VLP) in order to generate virus-neutralizing antibodies against 4 types of HPV (HPV 6, 11, 16, 18) [46]. VLP can be obtained by overexpression of the major capsid protein L1 alone, or by co-expression with the minor capsid protein L2 [47] [48]. HPV

VLP mimic infectious virions in structure and morphology [49] [50] and in their ability to induce high tittered neutralizing antisera, even in the absence of adjuvants [51]. Although VLP vaccine has been proven to be safe and effective for HPV infection, it cannot treat patients with established cervical neoplasia. Since capsid proteins are not detectably expressed in the basal epithelial cells that give rise to benign and malignant lesions, antibodies induced by VLP are unlikely to be active in treating established cervical cancer. There are a few studies that show vaccination with VLP generating capsid-protein specific cellular immune responses, however, such responses alone cannot result in regression of premalignant and malignant lesions in humans, but may aid in the prevention of such lesions [52] [53] [54].

### 1.2.3 Therapeutic vaccines against the existing cervical carcinoma

In order to cure patients who are currently HPV-infected, it is essential to develop a therapeutic vaccine for treatment of the HPV-associated lesions. Instead of L1 protein, HPV E6 and E7 oncoproteins are persistently expressed in the HPV infected cells including tumor cells. E6 and E7 proteins are typically intracellular nuclear antigens, but they also encode peptides that can serve as tumor rejection antigens [55]. The fact that these are foreign proteins and are not presented on the surface of the virus particle, makes them exquisitely tumor specific, and several of these antigens induce MHC class I-restricted CTLs. Indeed, active immunization against E6/E7 results in protection from tumor challenge [56]. Whereas tumors are dependent on the expression of E6 and E7, it provides a strong rational for targeting these proteins as a potential therapy for cervical cancer.

Therapeutic vaccines for treating cervical cancer should stimulate the immune system to recognize and directly target HPV viral proteins expressed in the infected cells, thereby

preventing the development of lesions and eliminating existing lesions [57] [58]. The choice of target antigen is a critical step for developing a potent therapeutic vaccine. Most therapeutic approaches have concentrated on eliciting cytotoxic T lymphocytes (CTL) responses against the constitutively expressed viral oncogenic antigens of the most prevalent high-risk HPV 16 and 18 viruses. Since endogenously derived viral peptides of E6 and E7 can traffic through the endoplasmic reticulum and Golgi apparatus and be presented on the surface of the infected cells, they are potential targets of recognition by $CD8^+$ CTL. In addition, E7 sequence is more conserved than that of other peptides and is expressed throughout the life cycle [59]. A number of animal studies listed below have indicated that therapeutic HPV vaccines targeting E6/E7 are effective in alleviating disease progression [60].

**Peptide-based vaccines**: A large set of both murine and human HPV-16 and HPV-18 CTL and helper T cell epitopes have been identified [61] [62]. Vaccination with E6/E7 peptide or lipidated peptides was found to generate CTL responses in both animals and human [63].

**Protein-based vaccine**: Whereas peptides exhibit MHC restriction, protein-based vaccines are independent on patient's HLA haplotype, thereby including all potentially immunogenicity of the targeted protein. Purified viral proteins induce both cellular and humoral immune responses including antibody and $CD4^+$ helper T cell responses. Due to the fast degradation of injected proteins, the use of adjuvants and multiple immunizations is generally required for a potent immune response [64].

**Life vector-based vaccines**: Live viral expression vectors such as vaccinia virus and adenovirus have also been used to generate HPV vaccines. The advantage of these vaccines is that HPV proteins are endogenously synthesized from the viral DNA by host cells, with the result that an array of HPV peptides is produced, processed and presented on the cell surface in

conjunction with the MHC class I molecule. Recombinant vaccinia expressing modified E6 and E7 antigens of HPV 16 and HPV 18 has been used successfully to provide protection against tumor growth in various systems [65] [66]. Adenovirus vectors have the advantage over vaccinia vectors of not inducing unwanted side effects associated with replication in mammalian cells and being engineered such that they are replication-deficient [67]. Attenuated bacteria such as Listeria and Salmonella can also serve as a carrier to deliver engineered plasmids encoding proteins of interest. Recent studies have shown that oral vaccination of Listeria monocytogenes that express HPV 16 E7 can lead to tumor regression in a mouse model.

**DNA-based vaccines**: DNA vaccines which allow for sustained expression of antigen is another type of vaccination strategy currently being explored for immunotherapy [68]. Immunization with naked DNA in conjunction with various strategies has been tested in various HPV16 tumor models [69] [70] [71].

**Chimeric VLP vaccines**: Chimeric HPV VLP that incorporate non-structural virus proteins such as E6 and E7 or peptide epitopes derived from such proteins offer an exciting approach for a combined prophylactic and therapeutic vaccine against HPV-induced lesions [72].

**Dendritic cell-based vaccines**: Bone marrow-derived DC pulsed with HPV 16/18 E7 peptide or tumor lysate were shown to serve as an effective vaccine, protecting mice against challenge with E7-expressing tumor cells and also causing regression of small existing tumors [73] [74].

### 1.2.4 Therapeutic vaccine in clinical trials

Several vaccines with safety, immunogenicity and clinical efficacy are currently being developed for clinical trials. Although there are no therapeutic vaccines against HPV available in the market

so far, several vaccines showed encouraging therapeutic results in the clinical trials (summarized in **Table 1.1**). A head-to-head comparison of these trials will help the design of a potent therapeutic vaccine with minimal toxicity [75]. The clinical trials also provide a good opportunity to investigate the mechanism of the immune response mediated by vaccine. This will greatly facilitate the rational design and development of improved therapeutic vaccines.

Most of the vaccines in the clinical trial utilized vaccine adjuvant or/and particulate delivery systems to enhance the immunological response. In a phase I clinical trial, 18 women with high grade HPV $16^+$ CIN were given an HLA-A2-resticted peptide ($E7_{12-20}$) in incomplete Freund's adjuvant. DC infiltrate was observed in 6 of 6 patients while CTL response was observed in 10 of 16 patients. Also, 3 out of 17 patients cleared their dysplasia after vaccination [76]. In a more recent clinic trial, Sheets et al. utilized pDNA encoding a 13 a.a. sequence (homologous with HPV E7) delivered by a poly lactide-co-glycolide microparticle (ZYC101) for a Phase I trial [77]. The results showed a 33% complete histologic responses, a 73% immunologic response, and no serious adverse events after treatment by ZYC101. These studies have demonstrated clearly the relevant biological and clinic effects of the HPV therapeutic peptide vaccines and, therefore, the potential for the management of the cervical cancer.

**Table 1.1. Clinical trials for therapeutic vaccines against cervical cancer**

| Antigen used | Delivery system | Study group | Outcome | Reference |
|---|---|---|---|---|
| pDNA (HPV 16 E7 peptide sequence) | Microparticles (ZYC101) | Phase I | E2 specific immunity | [77] |
| HPV 16/18 E6/E7 gene | Recombinant vaccinia virus | Late stage cervical cancer | Mild increase in antibody titer and CTL response | [78] |
| HPV 16 $E7_{11-20}$ peptide | Emulsion with Freund's adjuvant | Phase II/III: high-grade cervical or vulvar intraepithelial neoplasia | 10/16 had positive response and 12/18 cleared the virus | [76] |
| pDNA (HPV 16 E7) | Microparticles (ZYC101a) | High-degree anal intraepithelial lesions and high-grade CIN | 33% had complete response and 73% showed immunological response | [79] |
| pDNA (attenuated HPV 16 E7 fused to Hsp70) | - | HPV 16 positive head and neck cancer | Ongoing | [59] |
| $E7_{86-93}$ lipopeptide | - | Phase I: refractory cervical cancer or vaginal cancer | Positive CTL response but no clinical responses | [63] |
| Two HPV 16 E7 peptides plus one helper peptide | Emulsion with Montanide ISA51 adjuvant | Phase I/II: recurrent cervical cancer | 2/19 showed stable disease and 2/19 had tumor regression after chemotherapy | [80] |
| HPV 18 E7-pulsed dendritic cells | - | Patients with metastatic cervical cancer | Induction of tumor-specific cytotoxicity in tumor infiltrating lymphocytes | [81] |

## 1.3 CATIONIC LIPOSOME AS AN ANTIGEN CARRIER AND A CANCER VACCINE ADJUVANT

### 1.3.1 Peptide cancer vaccine and delivery system

Synthetic peptide-based vaccines designed to elicit T cell immunity are an attractive approach for the prevention and treatment of cancers. Tumor cells express antigens that can be recognized by the host immune system. These tumor-associated antigens (TAA), usually small peptides of 8-10 amino acids, can be injected into patient to induce immune responses that may result in the destruction of the cancer [82] [83]. Cytotoxic T lymphocytes recognize TAA that associate with MHC class I molecules expressed on the surface of the APC. As a result of the specific interaction between the TCR on the CTL and the peptide/MHC complex on the APC, the CTL are able to kill tumor cells expressing the TAA [84]. Over the last decade, numerous TAA recognized by tumor-reactive CTL have been identified by various molecular and biochemical methods [85]. However, most of them showed only limited clinical use for the majority of cancers. There are still many obstacles to overcome to take full advantage of the peptide cancer vaccines.

Firstly, the peptide antigen must make its way into the organized lymph tissue such as the draining lymph nodes (DLN) or the spleen [86]. This could be accomplished either through effective delivery of peptide directly to the lymphoid organs, or by effective targeting of peptide to antigen presenting cells (APCs) in the periphery, with concomitant delivery of appropriate "danger signals" (such as CpG motif) to induce APC maturation and migration. It has been

shown that large amounts of immunogenic antigens are ignored by the immune system, as long as they remain outside of the lymphatic system [87].

Secondly, to induce an effective cellular immunity, antigenic peptides in the lymphoid tissues must be presented to T-cells in association with MHC molecules on the surface of the APC such as DC. DC are the initiators and modulators of the immune response and as such can serve as an attractive target for vaccination strategies. Extracellular peptide antigens are generally vulnerable to protease due to the degradation of the minimal epitope. Even if they are internalization by the APC, it generally results in delivery to the lysosomal compartment, where the minimal epitope is rapidly degraded [88].

To circumvent these obstacles, a great number of systems have been designed to deliver antigenic peptides to the cytoplasm of the APC, from which they could access the MHC class I restricted processing pathway. The use of peptides associated with adjuvants or heat shock proteins, encapsulated in neutral liposomes or biodegradable polymers, or coupled to synthetic beads have all been investigated [89] [90]. Recently, Wang et al demonstrated that *ex vivo* loading of antigen into the cytoplasm of dendritic cells extends the time that it is presented on MHC class I molecules and enhances CTL responses [91].

### 1.3.2 LPD mediated peptide vaccination

Previous work in our lab has led to the development of a novel liposome-based delivery system, called LPD [92]. The LPD, originally designed as a DNA delivery system, is self-assembled from cationic lipid, protamine and plasmid DNA. LPD is also a novel adjuvant for stimulating both humoral and cellular immune responses. Intravenous administration of LPD was found to potentiate efficient anti-tumor activity in murine tumor model 24JK cells, even when the

formulation contained an empty, non-coding plasmid [93]. The observed anti-tumor activity was thought to originate from the innate immunostimulatory activity of the unmethylated CpG motif sequences present in the plasmid DNA. It was further correlated with its ability to induce Th-1 cytokines like TNF-α, IFN-γ and IL-12 and to the development of tumor-specific $CD8^+$ cytotoxic lymphocytes (CTL) [94].

Due to the feature of immunogenicity, LPD particles were used to entrap and deliver antigenic peptides to the cytoplasm of APC, thereby enhancing the immune response to peptide vaccines. When LPD loaded with a MHC class I-restricted peptide (RAHYNIVTF) epitope derived from E7 protein of HPV-16 was used to immunize the mice, a strong antigen-specific anti-tumor response was observed [95]. It induced an E7-specific CTL response and prevented the establishment of E7-expressing TC-1 tumors, a murine model for human cervical cancer. Moreover, administration of LPD/E7 to TC-1 tumor-bearing mice caused complete tumor regression. Flow cytometry analysis with fluorescently labeled complexes revealed that LPD/E7 particles are selectively accumulated in the spleen or the local nymph nodes and are effectively taken up by the antigen presenting cells of the immune system. Furthermore, a murine dendritic cell line (DC2.4) pulsed with LPD/E7 complex could effectively induce an E7-specific CTL response and immunize the mouse against tumor challenge [96]. These data indicated that LPD is an excellent carrier for peptide antigen for inducing CTL response against $HPV^+$ tumor.

### 1.3.3 Vaccine adjuvant activity of cationic liposome

To elucidate the immunostimulatory mechanism of LPD and to further improvise its adjuvant activity, we investigated a series of detailed mechanistic studies of the stimulatory activity of LPD on DC2.4 and bone marrow derived dendritic cells (BMDC). We observed that the cationic

lipid part of the LPD formulation significantly influences its immunostimulatory activity on DC. Our data show DC activation correlates with the concentration of cationic lipid [97]. The fact that cationic lipids alone could stimulate the antigen presenting cells was of high importance since the immunomodulatory adjuvant activities of LPD were initially thought to be arising only by unmethylated CpG motifs of the plasmid DNA via toll-like receptor 9 (TLR9). Recent studies have shown that bacterial CpG motifs may cause acute inflammatory response in humans, thus, it is essential to develop a safer vaccine formulation for human use which contains no bacterial DNA.

The immunologic adjuvant property of liposomes was first studied by Allison and Gregoriadis [98]. Their results showed that diphtheria toxoid entrapped in liposomes generated a strong humoral immune response in mice. Uemura et al. [99] found that liposomes prepared from various phospholipids had differential adjuvant activity. The lipid structure determines the properties of liposomes including the membrane stability, surface charge, and to some extent, the interior properties of the liposome. Therefore, we have systematically investigated structure-specific immunomodulatory effect of cationic lipids on DC [97]. Our data show some of the cationic lipids alone can activate dendritic cells toward expression of surface marker, CD80 and CD86, but not the release of TNF-α from DC. DC stimulation was specific to cationic lipids, the zwitterionic and anionic lipids showed little or no activity. In general, the cationic lipids bearing ethyl phosphocholine head groups were better stimulants than their trimethylammonium counterparts. These results suggest the existence of an NF-κB independent immunostimulation pathway for cationic lipids such as DOTAP.

More recently, we found that cationic liposomes, such as DOTAP, can stimulate DC, resulting in MAP kinase ERK activation and several chemokine inductions, such as CCL-2 [100].

A proposed signaling pathway of DOTAP is summarized in **Fig. 1.1**, which reveals a molecular mechanism of DOTAP as a vaccine adjuvant. PI-3 kinase is required for DOTAP-induced CCL2 production and ERK phosphorylation. pERK and CCL2 production in DC are partially inhibited when PI-3 inhibitor (i.e. wortmannin) is added. In addition, ERK activation and CCL2 release by DOTAP is negatively regulated by p38 pathway. Interestingly, the anti-tumor activity of DOTAP/E7 vaccine is significantly decreased when either U-0126 (ERK inhibitor) or SB203580 (p38 inhibitor) is co-formulated with the vaccine. The result implies that both ERK and p38 pathway are required by DOTAP-mediated vaccine adjuvant activity [100]. Although the detail mechanism is not fully elucidated, our data suggest that DOTAP adjuvant-induced DC activation is different from other type of adjuvants which activate DC through TLR signaling, leading to NF-κB production.

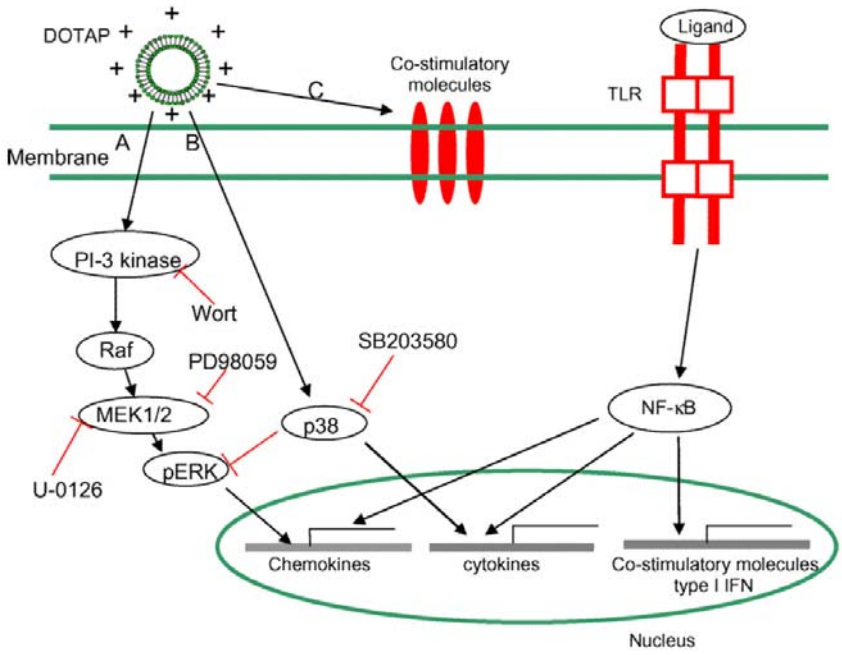


**Figure 1.1. (adopted from Yan, 2007) Proposed signaling pathways leading to DC activation and expression of chemokines/cytokines induced by DOTAP liposomes.**
(A) DOTAP triggers ERK activation through PI-3 kinase, inducing CC chemokine expression; (B) DOTAP also activates p38, which negatively regulates the ERK activation and also results in Th1 cytokine, such as IL-12 expression. (C) Through unknown mechanisms, DOTAP induces co-stimulatory molecules expression and DC activation. NF-κB activation mediated by TLR is also plotted to compare with DOTAP induced signal transduction.

### 1.3.4 Investigation of current DOTAP/E7 cancer vaccine

The encouraging results above prompted us to initiate a series of studies to assess whether cationic DOTAP liposome by itself could function as an immune stimulator such that an effective vaccine could be formulated. DOTAP/E7, which contains only an antigen and a cationic lipid was then prepared and studied in our lab. The physicochemical properties and anti-cancer effect of DOTAP/E7 was compared side by side to those of LPD/E7, the previous vaccine version. A plausible mechanism to initiate DOTAP-mediated DC activation was also discussed.

In addition, we further improved the vaccine formulation by incorporation of E7-lipopeptide instead of the water-soluble native E7 peptide into the DOTAP liposome. The anti-tumor activity of lipopeptide formulated in DOTAP liposome was more than twice as potent as that of native E7, likely owing to the increased peptide entrapment efficiency in the liposomal complex.

Finally, we tested our vaccine formulation on the human model system. Instead of murine H-2D$^b$ restricted peptide, a HLA-A2 restricted CTL peptide epitope derived from E7 protein was formulated in the liposome. The results of DOTAP/hE7 vaccine obtained from HLA-A2 transgenic mice or human blood cells were consistent to those collected from mouse model system.

Overall, the report herein suggests that DOTAP/E7 is a potent therapeutic cervical cancer vaccine formulation which can be used in several clinical applications for the treatment of HPV-related neoplasia.

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## 2.0 A SIMPLE BUT EFFECTIVE CANCER VACCINE CONSISTING OF AN ANTIGEN AND A CATIONIC LIPID

Developing a cancer vaccine with a potent adjuvant, which is safe for human use, remains to be an unmet challenge. In this study, we developed a simple, safe, yet efficient, peptide-based therapeutic cancer vaccine, DOTAP/E7 complex, which comprises only two molecules: a DOTAP cationic lipid and a peptide antigen derived from E7 oncoprotein of human papillomavirus (HPV) type 16. The anti-cancer activity of DOTAP/E7 against existing HPV positive TC-1 tumor was compared to that of our previous LPD/E7 formulation, which contains bacterial DNA CpG motifs. Tumor-bearing mice showed significant tumor inhibition following a single vaccination of either formulation at the optimal lipid dose, suggesting that DOTAP liposome alone can provide a potent adjuvant activity without plasmid DNA. E7 peptide formulated with DOTAP induced migration of activated dendritic cells (DC) to the draining lymph node (DLN) and efficiently generated functional antigen-specific $CD8^+$ T lymphocyte responses. Accumulation of $CD8^+$ tumor infiltrating T cells and apoptosis at tumor sites were observed after treatment with DOTAP/E7 complexes, which was also associated with a decreased amount of $CD25^+Foxp3^+$ regulatory T cells in treated animals. Reactive oxygen species (ROS) induced by DOTAP cationic lipid in DLN revealed a plausible mechanism of the initial interaction between DC and DOTAP. An adequate amount of ROS generation was apparently required for the initiation of the DOTAP/E7 vaccine action; however, an overdose of DOTAP induced a massive ROS production and apoptosis of DC in DLN, which led to a

diminished anti-cancer immunity. Overall, these results indicate that the cationic lipid DOTAP alone serves as an efficient vaccine adjuvant for the induction of a therapeutic, antigen-specific anti-cancer activity.

## 2.1 INTRODUCTION

The development of safe and effective therapeutic cancer vaccines for human use remains an urgent and unmet medical need. In order to elicit appropriate protective immune responses, the use of immunologic adjuvant to enhance and direct the immune response is required in a rational vaccine design [1]. For vaccines composed of synthetic peptide or subunit antigen, which are often unable to induce adequate immune responses, an appropriate adjuvant is necessary to improve the immune response and process a weakly immunogenic antigen. An ideal adjuvant for a therapeutic cancer vaccine should: increase the biological or immunological half-life of the vaccine antigen; improve antigen delivery and/or processing in the antigen presenting cells (APC) [2]; reduce the number of immunizations or the amount of antigen required for an effective vaccine [3]; induce the production of immunomodulatory cytokines that favor the development of Th1 immune responses to the vaccine antigen, thus promoting cell-mediated immunity including CTL [4]; and finally, overcome immune tolerance to tumor by inhibiting immune suppressive factors [5].

Previous work in our lab has led to the development of a liposome-based, nanoparticle delivery system, called LPD [6]. LPD, originally designed as a gene delivery system, is self-assembled from cationic liposomes, polycations and plasmid DNA. LPD is also a novel vaccine adjuvant for stimulating both humoral and cellular immune responses [7]. Intravenous

administration of LPD potentiates efficient anti-tumor activity in a murine tumor model 24 JK, even when the formulation contained an empty, non-coding plasmid [8]. It was further correlated with its ability to induce Th-1 cytokines such as TNF-α, IFN-β and IL-12 and to the development of tumor-specific $CD8^+$ cytotoxic lymphocytes [9]. The observed anti-tumor activity originates from the innate immunostimulatory activity of the unmethylated CpG motif, abundantly present in the plasmid DNA [10]. Cationic liposomes are capable of protecting plasmid DNA from extracellular degradation and thus more DNA enters the endosomal compartment where the toll-like receptor 9 (TLR9) is selectively expressed [11], [12]. However, cationic liposomes alone are relatively inert in terms of activating immune responses and there have been no studies so far to support that the cationic liposome by itself can serve as an efficient adjuvant [13], [14].

Our previous studies have shown that a strong antigen-specific anti-tumor response was observed in mice, which received LPD particles loaded with an MHC class I-restricted peptide (RAHYNIVTF) epitope derived from the E7 protein of human papillomavirus (HPV) type 16 [15], [16]. It induced an E7-specific CTL response, which contributed both to protective and therapeutic effects against E7-expressing TC-1 tumors, a murine model for human cervical cancer [17]. Recently, we have also investigated structure-specific immunomodulatory effects of cationic lipids on dendritic cells [18]. Our data suggest that some of the cationic lipids alone can activate dendritic cells toward expression of surface markers, CD80 and CD86. Moreover, we found that cationic liposomes, such as DOTAP, could stimulate DC, resulting in MAP kinase ERK activation and chemokine induction, such as CCL-2 [19]. The fact that cationic lipids alone could stimulate the antigen presenting cells was of high importance, since the

immunomodulatory adjuvant activities of LPD were initially thought to arise only by unmethylated CpG motifs of the plasmid DNA via the TLR9 [20], [21].

The results prompted us to initiate a series of studies to assess whether cationic DOTAP liposome by itself could function as an immune stimulator such that an effective vaccine could be formulated. In the present study, we examined for the first time the immunological mechanism and anti-cancer effect of an efficient therapeutic cancer vaccine formulation, DOTAP/E7, which contains only an antigen and a cationic lipid. We describe herein the anti-tumor activity of this simple vaccine.

## 2.2 MATERIALS AND METHODS

### 2.2.1 Lipids, reagents and murine tumor cell lines

DOTAP and other lipids were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). Protamine sulfate was purchased from Sigma-Aldrich (St. Louis, MO, USA) and plasmid DNA (pNGVL3) was obtained from the National Gene Vector Laboratory (Ann Arbor, MI, USA). CpG ODN1826 was requested from IDT, Inc. (Coralville, IA, USA) and the complete Freund's adjuvant (CFA) was purchased from DIFCO Laboratories (Detroit, MI, USA). Murine TC-1 cells were kindly provided by Dr. T.C. Wu at Johns Hopkins University (Baltimore, MD, USA). TC-1 cells are C57BL/6 mouse lung epithelial cells transformed with HPV 16 E6 and E7 oncogenes and the activated H-ras. BL6 are melanoma cells derived from C57BL/6. Both murine tumor cell lines were maintained in RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA)

supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin (Invitrogen).

### 2.2.2 Peptide synthesis

H-2D$^b$ restricted peptide RAHYNIVTF derived from HPV 16 E7 protein (amino acid 49-57) was synthesized and purified in Molecular Medicine Institute Peptide Synthesis Facility in the University of Pittsburgh.

### 2.2.3 Preparation and characterization of DOTAP/E7 and other liposomal complexes

Cell culture grade water (Cambrex, Walkersville, MD, USA) was used in all liposome preparation procedures. Briefly, lipid films were made in a glass vial by evaporating the chloroform solution under a steady stream of dry nitrogen gas. Traces of organic solvent were removed by keeping the films under vacuum with desiccators overnight. Lipid films were hydrated for 12 h by adding the required amount of water to make a final concentration of 10 mg/ml. The suspensions were then sonicated in a bath-type sonicator for 10 min followed by extrusion (Hamilton Co., Reno, NV, USA) through 400, 200 and 100 nm membrane filters and were stored at 4°C before use. For the preparation of DOTAP/E7, the lipid film was hydrated with an aqueous solution of E7 peptide instead of water. LPD formulation comprises DOTAP lipid, protamine and plasmid DNA at a ratio of 8.6:0.6:1 (w/w/w). LPD was made as described [16] and the complex was allowed to stay at room temperature for at least 20 min prior to the injection.

The particle size and the zeta (ζ) potential of the liposomal complexes were measured following the manufacturer's instruction using a Coulter N4 Plus particle sizer (Beckman Coulter, San Francisco, CA, USA) and a Zeta-Plus (Brookhaven Instruments, Corp., Holtsville, NY, USA). Peptide encapsulation was determined by the percentage of liposome-bound peptide. In brief, the unbound peptide from DOTAP/E7 and LPD/E7 was separated from the complex by a Microcon® centrifugal filtrate device (Millipore, Bedford, MA, USA) and the concentration of unbound peptide was measured by Micro BCA™ protein assay kit (Pierce, Rockford, IL, USA). The efficiency of peptide encapsulation was determined as (1- % unbound peptide) and was reported as mean ± SD (n =3).

### 2.2.4 Mice and immunizations

All work performed on animals was in accordance with and permitted by our institutional IACUC. C57BL/6 female mice, 6-7 weeks old were purchased from Charles River Laboratories (Wilmington, MA, USA) and were used in all animal studies. Subcutaneous tumors were established by injecting TC-1 cells ($10^5$ cells) into the hair-trimmed flank of the mouse on day 0. On day 6, mice (n = 6-12) were injected subcutaneously (sc) at the opposite side of the flank with 150 μl of selected formulations containing 10 μg of the E7 peptide. The size of the tumor was measured using a caliper two or three times a week. Tumor size was determined by multiplying the two largest dimensions of the solid tumor.

### 2.2.5 Antibodies and flow cytometric analysis

All anti-mouse antibodies used for flow cytometric analysis were purchased from BD Pharmingen (San Diego, CA, USA) or eBioscience, Inc. (San Diego, CA, USA). Spleen cells or lymph node cells were harvested and dissociated by incubating with 1 mg/ml collagenase (Sigma-Aldrich) for 20 min and passing through a 70 μm cell strainer (BD Biosciences). After removal of red blood cells (RBC), the cell suspensions were incubated with anti-CD16/CD32 (24.G2) on ice for 15 min to block non-specific binding, followed by immunostaining with fluorescently conjugated antibodies to surface antigens for 30 min at 4°C. The following Abs were used: anti-CD3e (145-2c11), anti-CD4 (RM4-5), anti-CD8a (53-6.7), anti-CD11c (HL3), anti-CD25 (pc61.5), anti-CD80 (16-10A1), anti-CD86 (GL1) and anti-NK1.1 (pk136). Isotype control Abs were used to set the background for the surface Ab labels. Prior to staining intracellular molecules such as Foxp3 and IFN-γ, cells were fixed and permeabilized using the Cytofix/Cytoperm™ kit (BD Pharmingen) according to the manufacturer's instruction. Cells were finally resuspended in 300 μl stain buffer and analyzed using a BD FACSCanto digital flow cytometer (San Diego, CA, USA).

### 2.2.6 Tracking uptake of fluorescent DOTAP/E7 complexes *in vivo*

The mice were sc injected with DOTAP/E7 containing 100 nmol total lipid with 0.05% (w/w) NBD-DOTAP. The draining lymph nodes (DLNs) were isolated at various time points after injection. Cell suspensions were stained with appropriate antibodies and analyzed by flow cytometry. Analysis gates were set on live cells based on the forward and side scatter characteristics.

### 2.2.7 Immunohistochemistry and TUNEL

Solid TC-1 tumors were established as described and the mice were given treatments on day 6. On day 14, tumor samples were dissected and embedded in Tissue-Tek® OCT compound (Sakura Finetek, Torrance, CA, USA) followed by cryosection preparation. The samples were cut into 8 μm thick sections with a cryostat (Hacker Instruments & Industries Inc., Winnsboro, SC, USA). The sections were stained with FITC-conjugated anti-CD8 or anti-CD4 antibodies (Miltenyi Biotec Inc. Auburn, CA, USA) to determine tumor-infiltrating T cells. The samples were mounted on a coverslip using Vectashield® mounting solution (Vector Laboratories, Inc., Burlingame, CA, USA) containing DAPI to counterstain nuclei. Images of the sections were taken using a Leica SP2 confocal microscope.

TUNEL assay was conducted using a TACS™ TdT Kit (R&D Systems, Minneapolis, MN, USA) and developed with DAB according to manufacturer's instructions. Samples were imaged using a Nikon Microphot SA microscope.

### 2.2.8 Analysis of Ag specific CTL and immune response

Naïve C57BL/6 mice were immunized with 10 μg E7 peptide formulated in DOTAP liposomal formulations on day 0 and 7. For *in vitro* CTL assay, the mice were killed 7 days after the last immunization and spleen cells were isolated and dissociated. After RBC lysis, the total spleen cells (responder cells) were *in vitro* stimulated for 5 days with E7 peptide (10 μg/ml) in the presence of β-mercaptoethanol and 40 U/ml recombinant mIL-2 (R&D Systems) in complete RPMI-1640 medium. After 5-day of CTL expansion, responder cells were used as CTL effectors while TC-1 tumor cells were used as target cells in this assay. To discriminate between effectors

and targets, TC-1 cells were labeled with PKH-67 (Sigma-Aldrich) according to manufacturer's instructions. The mixture of effectors and labeled targets were plated into 96-well U-bottom plates at various effector:target (E:T) ratios and the cell lysis reactions were carried out for 4 h at 37°C. The cells were then harvested and stained with propidium iodide (Sigma Aldrich) prior to flow cytometric analysis. The percentage of E7-specific lysis was determined by the proportion of PI positive cells within the FL1 (PKH-67) positive region.

*In vivo* CTL activity of E7-specific cytotoxic T cells was enumerated according to the protocol of Byers et al. [22] with minor modifications. In brief, spleen cells from syngenic mice were RBC lysed followed by pulsing with 10 μM E7 peptide or without peptide in complete medium for 1 h at 37°C. Both spleen cell populations were stained with equal amount of 2 μM PKH-26 (Sigma-Aldrich) according to manufacturer's instruction. The peptide pulsed and unpulsed populations were loaded with 4 and 0.4 μM CFSE (Molecular Probe), respectively, at 37°C for 15 min. The two cell populations were mixed together (1:1) before tail vein injection to the control or DOTAP/E7 immunized mice ($10^7$ cells per mouse injection). At 16 h after injection, spleen cells from the recipient mice were isolated and single cell suspension were prepared prior to flow cytometric analysis. The number of $CFSE^{high}$ and $CFSE^{low}$ population were determined and the *in vivo* E7 specific lysis percentage was enumerated according to a published equation [22].

For the measurement of IFN-γ producing $CD8^+$ T cells, spleen cells were isolated from the control or immunized mice 7-10 days after the last immunization. $2 \times 10^6$ spleen cells were incubated with 5 μg/ml E7 peptide or without peptide in the presence of 1 μl/ml of GolgiStop™ (BD Pharmingen) for 6 h. After washing with FACS buffer, cells were stained with Abs to

surface markers and intracellularly with IFN-γ mAb (XMG1.2) prior to analysis by flow cytometry.

### 2.2.9 Analysis of ROS cytotoxicity in the DLN

The mice were sc injected with DOTAP/E7 containing 0, 15, 100 or 600 nmol total lipid and the DLNs were isolated at a 2 h time point after injection for the analysis of ROS production. Single cell suspensions were prepared and incubated with 20 μM 2', 7'-dichlorodihydrofluorescein diacetate (DCFH-DA) (Sigma-Aldrich) in serum free medium in the dark for 30 min at 37°C. The non-fluorescent DCFH-DA readily diffuses into the cells where it is hydrolyzed to become the polar derivative DCFH, which is oxidized in the presence of ROS to the highly fluorescent 2', 7'-dichlorofluorescein (DCF) [23]. The cells were quickly washed with pulse spin and immediately analyzed by flow cytometry. For determining cytotoxicity induced by the high dose of DOTAP lipid, DLNs from the immunized mice were collected at 10 h after injection, followed by subsequent staining with Abs to the surface markers. Cell suspensions were added with propidium iodide prior to flow cytometric analysis. The percentage of cell death of DC in the DLN was determined by the proportion of $PI^+$ cells within the $CD11c^+$ region.

### 2.2.10 Statistical analysis

Data were analyzed statistically using a two-tailed Student's t-test. Data were considered statistically significant when the P value was less than 0.05.

## 2.3 RESULTS

### 2.3.1 Cationic DOTAP liposome as a potent cancer vaccine adjuvant, which provides a major contribution toward the anti-tumor activity of LPD vaccine formulation

Previous investigations by our group showed that LPD encapsulated with an MHC class I peptide epitope derived from HPV (type 16) E7 protein can generate a preventive as well as a therapeutic effect against the HPV16 E7 positive tumor cell line, TC-1 [17], [16]. However, there are concerns regarding LPD/E7 vaccine formulation such as the possible toxicity, which can be induced by a high dose of lipid-DNA complex (lipoplex) [24]. To assess the adjuvant activity of lipoplex, we examined anti-TC-1 tumor activity of LPD/E7 formulation at various DOTAP lipid concentrations. TC-1 tumor bearing mice sc received 10 μg E7 peptide formulated in LPD on day 6 post tumor inoculation (**Fig. 2.1a**). DOTAP lipid concentrations in LPD varying from 3 to 600 nmol were investigated and the L:P:D weight ratio (8.6:0.6:1) was kept the same in all groups. The mice that received LPD/E7 containing the lowest amount of DOTAP (3 nmol) showed partial tumor inhibition ($P < 0.05$) compared to the untreated control group on day 23. LPD/E7 with DOTAP at 15 or 30 nmol exhibited an enhanced efficacy ($P < 0.01$), while LPD/E7 with 75 or 150 nmol lipid showed the most significant tumor regression ($P < 0.001$). Interestingly, mice given high doses of LPD (300 or 600 nmol) did not show significant tumor inhibition.

Upon receiving cationic lipoplex, bacterial DNA containing immunostimulatory CpG motif activates host innate immune response, which includes the induction of proinflammatory cytokines and immune cell activation, especially in human [25]. To avoid the drawbacks of CpG effect and to assess whether cationic lipid alone could function as a cancer vaccine adjuvant, we

have investigated a new formulation that only contains cationic DOTAP liposomes and the E7 peptide. The new formulation, DOTAP/E7, without plasmid DNA and protamine sulfate, also exhibited anti TC-1 tumor effect (**Fig. 2.1b**). The tumor growth kinetics in mice treated with DOTAP/E7 was similar to that of the LPD/E7 formulation. Low dose of DOTAP (15 nmol) showed partial TC-1 tumor inhibition effect ($P < 0.05$) compared to the untreated control on day 23, while DOTAP at 30, 150 or 300 nmol exhibited an enhanced efficacy ($P < 0.01$). DOTAP at 75 nmol showed the most significant tumor regression effect ($P < 0.001$). Again, mice given a high dose of DOTAP (600 nmol) did not show anti-tumor activity, suggesting that DOTAP liposomes at a high dose might have induced a negative regulation to the immune response. In addition, DOTAP liposomes at optimal dose, but without E7 peptide, did not show significant inhibition, indicating that the anti-tumor effect was antigen specific. Anti TC-1 tumor activity of both DOTAP/E7 and LPD/E7 formulations was compared on day 23 by plotting the tumor size at each corresponding DOTAP lipid concentration (**Fig. 2.1c**). The data is pooled from three independent experiments and it is evident that these two dose response curves are almost identical to each other and no significant difference was found in any paired tumor size comparison. This result suggests that the DOTAP liposome is the major active ingredient in the LPD formulation to provoke immune response. Plasmid DNA and protamine are not necessary for the potent anti-tumor activity of the vaccine. We have also shown that low or non-detectable TNF-$\alpha$ production (<10 pg/ml) in the sera of mice injected with DOTAP/E7, indicating low toxicity of our vaccine formulation. Also, according to the bell-shape curves in **Fig. 2.1c**, the optimal dose of DOTAP liposome is around 100 nmol and both low and high doses compromise its anti-tumor activity. This finding tempted us to investigate the role of DOTAP as a vaccine adjuvant in supporting the anti-cancer activity of DOTAP/E7.

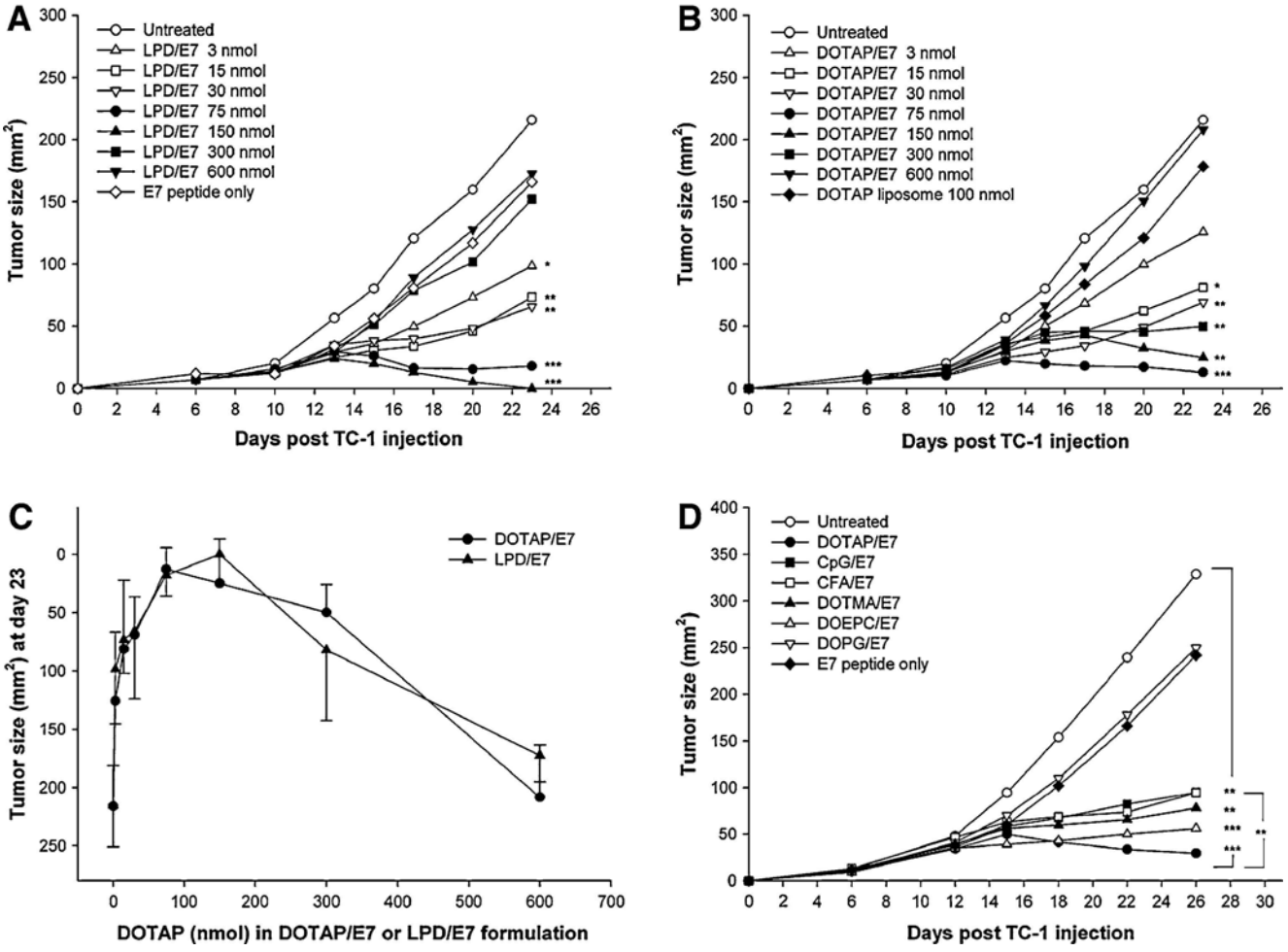


**Figure 2.1. Kinetics of TC-1 tumor growth in mice treated with LPD/E7 or DOTAP/E7 formulation.**
A, C57BL/6 female mice of 7 weeks old (n = 8-12) were injected sc with TC-1 tumor on day 0. On day 6, the mice received 10 μg E7 peptide formulated in LPD at various DOTAP lipid concentrations. The untreated mice were used as a negative control. B, On day 6 post TC-1 inoculation, the mice received treatment of 10 μg E7 peptide formulated in DOTAP liposomes at various lipid concentrations. Tumor sizes were measured with calipers and determined by multiplying the two largest dimensions of the tumor. Tumor size of each group on day 23 was compared to the untreated control group and was analyzed statistically (*P <0.05, **P < 0.01, ***P <0.001). C, The anti TC-1 tumor activity of the formulations DOTAP/E7 and LPD/E7 was contrasted on day 23 by comparing the tumor size at each corresponding DOTAP lipid concentration. No statistically significant differences in tumor size were found between the two groups. D, Anti-cancer activity of DOTAP/E7 was compared to that of other cationic lipids (DOEPC and DOTMA), an anionic lipid (DOPG), CFA and CpG ODN1826. Tumor-bearing mice (n = 6-12) received a single treatment on day 6 after tumor inoculation. TC-1 tumor sizes were measured and analyzed statistically.

To assess the efficacy of DOTAP/E7-induced immune response compared to other adjuvants, tumor-bearing mice were treated on day 6 with E7 peptide formulated in DOTAP, two additional cationic liposomes, DOEPC and DOTMA, an anionic lipid, DOPG, CFA and CpG ODN1826. In **Fig. 2.1d**, the mice that received the above formulations showed significantly smaller tumor sizes compared to the untreated group on day 26, except for those that received DOPG/E7, which did not show tumor regression. More importantly, the mice that received DOTAP/E7 formulation exhibited a better anti-cancer activity ($P < 0.01$) compared to those that received CpG/E7 or CFA/E7 formulations.

### 2.3.2 Physical properties of DOTAP/E7 complexes and the efficiency of E7 peptide entrapment in the liposome

DOTAP liposomes and DOTAP/E7 particles were prepared at 0.1 mM and their particle sizes and zeta ($\zeta$) potentials were characterized. The particle size of DOTAP/E7 was not significantly changed compared to that of the plain DOTAP liposome, which was 100 ± 24 nm in diameter. Also, the zeta potential of DOTAP/E7 particle was similar to that of DOTAP liposomes (43.5 ± 7.8 mV), indicating that the particle remains positively charged after changing the composition of DOTAP lipid and peptide. E7 peptide entrapment efficiency in DOTAP/E7 particles, which was determined by the percentage of liposome-bound E7 peptide was around 32 ± 4% in both DOTAP/E7 containing 100 or 600 nmol total lipids, whereas DOTAP/E7 with 15 nmol lipid showed a lower entrapment efficiency (17 ± 4%).

### 2.3.3 DOTAP/E7 particles are mainly taken up by dendritic cells after sc injection, resulting in the migration of activated DC to the DLN

Previous studies from our lab describe the ability of cationic liposome to activate mouse bone marrow dendritic cells *in vitro* [18], and the production of IL-12 by BMDC increases in response to DOTAP concentration (data not shown). The findings prompted us to hypothesize that DC, the professional APC, would be the direct responder after sc injection of DOTAP/E7 vaccine *in vivo*. To address the effect of DOTAP/E7 on DC activation and migration to the DLN, naïve mice were injected sc with DOTAP/E7 containing 0.5% (w/w) NBD-DOTAP at the flank. DLNs were harvested and analyzed by flow cytometry at different time points, post injection. The numbers of $CD11c^+$ cells among total lymph node cells were increased more than 2.5-fold compared to the untreated at 4 h after NBD-DOTAP/E7 injection (**Fig. 2.2a, b**). The expression of costimulatory molecule CD86 on the $NBD^+$ cells was investigated. $NBD^+$ cells demonstrated high levels of CD86 (**Fig. 2.2c, d**), indicating that sc injection of DOTAP prompted DC activation. NBD uptake by other cell types such as T lymphocytes was also investigated by co-staining with anti-CD3, CD4 and CD8 Abs. The dot plots in **Fig. 2.2e, f** were gated on the $CD3^+$ population. Neither of the $CD8^+$ or $CD8^-$ cells showed NBD uptake after NBD-DOTAP injection. The results clearly demonstrated that NBD-DOTAP is mainly taken up by DC (80%) soon after immunization and DOTAP induces migration of activated DC to the DLN, resulting in DC-T cell interactions and eliciting T cell responses.

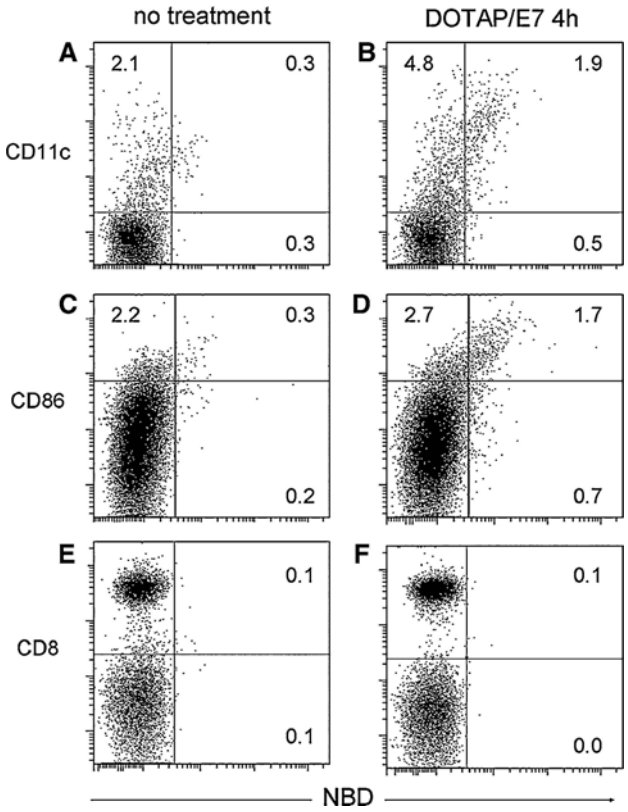


**Figure 2.2. Subcutaneous injection of DOTAP induces DC activation and migration to the DLN.**
Naïve mice (n = 4–6) were injected sc with PBS control (A, C, E) or DOTAP/E7 containing 100 nmol total lipid with 0.5% NBD-DOTAP (B, D, F). At 4 h after the injection, DLNs were prepared and stained with appropriate antibodies to surface markers. The co-expression of NBD and CD11c (A, B) or CD86 (C, D) was analyzed within total lymph node cells, whereas the co-expression of NBD and CD8 (E, F) were gated and analyzed within the $CD3^+$ population. The numbers represented the percentages of cells in the quadrants.

### 2.3.4 Tumor infiltrating T lymphocytes were found in mice treated with DOTAP/E7 at an optimal lipid dose

To understand whether the optimal DOTAP/E7 formulation induces migration of T lymphocytes to the tumor microenvironment, we performed immunohistochemistry staining on the frozen tumor tissues to examine tumor-infiltrating T lymphocytes (**Fig. 2.3**). TC-1 tumor-bearing mice were treated with PBS, DOTAP/E7 (containing 15, 100 or 600 nmol lipid) or LPD/E7 as described. On day 14 after TC-1 inoculation, solid tumors were harvested for preparation of cryosection and immunostaining. An increased amount of $CD8^+$ T lymphocytes (~5%) were

found in mice that received LPD/E7 (data not shown) or DOTAP/E7 at the optimal lipid dose (100 nmol, **Fig. 2.3b**) compared to untreated (**Fig. 2.3a**) or those received DOTAP overdose (600 nmol, **Fig. 2.3c**). A similar result was found for $CD4^+$ T cells; around 2% of tumor infiltrating $CD4^+$ T lymphocytes were found in mice that received an optimal dose of DOTAP (**Fig. 2.3e**), but not in the untreated or overdosed group.

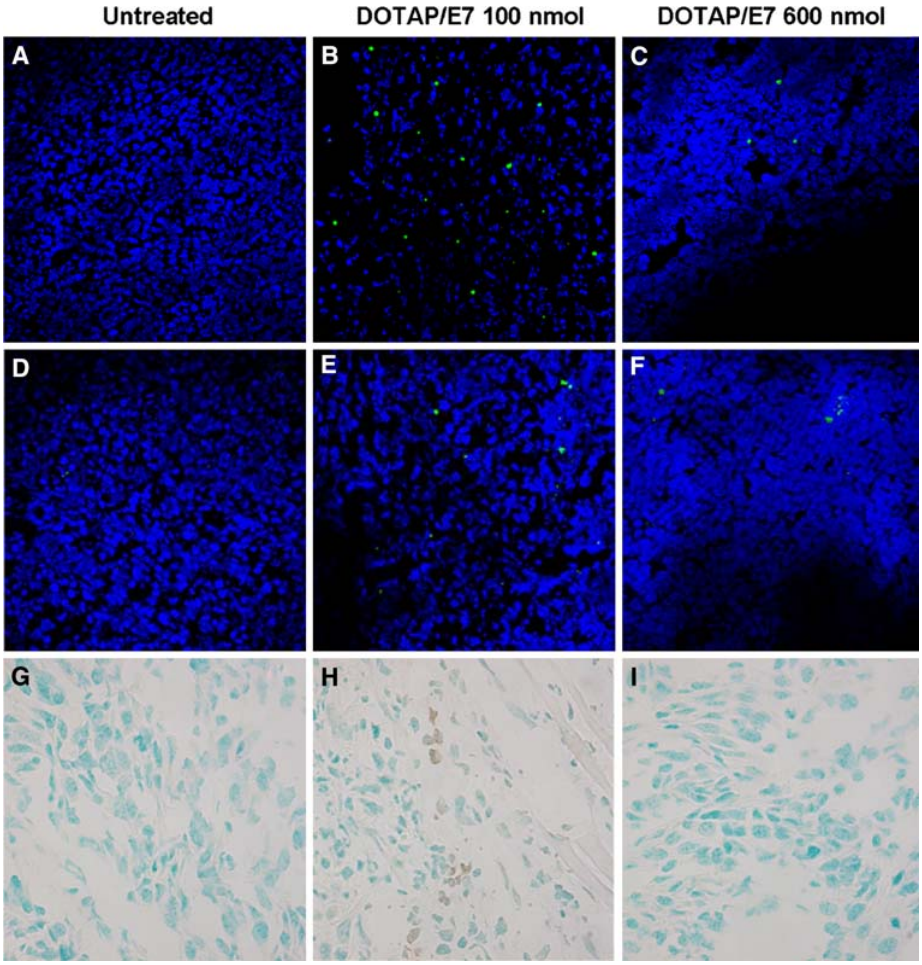


**Figure 2.3. Tumor infiltrating T-lymphocytes were found in mice that received DOTAP/E7 at an optimal dose.** TC-1 tumors were established as described and were left untreated or given treatment on day 6. Solid tumors were dissected on day 14 and examined for infiltrating lymphocytes. FITC conjugated anti-CD8 (A, B, C) and anti-CD4 (D, E, F) antibodies were used to determine tumor infiltrating T cells followed by counterstaining with DAPI. Representative tumor sections from groups of three mice were examined as described and imaged by confocal microscopy. TUNEL assay was conducted to detect cell apoptosis in the tumor sections (G, H, I).

TUNEL assay for determining apoptotic cells was also performed in tumor cryosections. A TUNEL-positive reaction was observed in the condensed and fragmented nuclei of the tumor cells after the mice were treated with LPD/E7 (data not shown) or DOTAP/E7 at an optimal dose (**Fig. 2.3h**) compared to the untreated (**Fig. 2.3g**) and overdosed group (**Fig. 2.3i**), which exhibited normal and vital tumor cells. Similar result, from Hematoxylin and Eosin staining (data not shown), showed that tumor necrosis was found only in the mice treated with E7 formulated with an optimal dose of DOTAP. The results indicate that the eliciting $CD8^+$ T lymphocytes were attracted to the tumor micro-environment and tumor cells were undergoing apoptosis or necrosis upon DOTAP/E7 treatment.

### 2.3.5 Decreased amount of Treg population in DOTAP/E7 treated mice was correlated with the anti-tumor activity

Despite intense recent interest, the suppressive mechanisms of regulatory $CD4^+CD25^+$ T cells (Tregs) remain poorly understood. Tregs are thought to dampen functional T cell immunity to tumor-associated antigens and to be the main obstacle, hampering successful immunotherapy and active vaccination [26]. It becomes necessary to monitor and characterize both effector and Treg responses in hosts that receive candidate tumor vaccine [5]. In **Table 2.1**, ten representative experiments are summarized on the phenotype analysis for the spleen cells obtained from tumor-bearing mice after vaccine treatment. The population of $CD4^+$ T cells was significantly increased in spleen cells from mice that received DOTAP/E7 treatment with an optimal lipid dose. The percentage of $CD4^+$ within the total lymphocyte population was shown. An increased amount of $CD8^+$ T cells was also observed (data not shown).

**Table 2.1. Treg subpopulation profile in spleen cells obtained from tumor-bearing mice after treatments with liposomal vaccine**

| | $CD4^+$ in lymphocytes | $Foxp3^+$ in CD4+ cells | $CD25^+$ $Foxp3^+$ in $CD4^+$ cells |
|---|---|---|---|
| PBS treated | 18.8 ± 2.9% | 13.1 ± 0.1% | 9.5 ± 0.5% |
| DOTAP/E7 15 nmol | 21.7 ± 1.3% *** | 12.0 ± 1.0% ** | 8.4 ± 0.2% *** |
| DOTAP/E7 100 nmol | 25.1 ± 2.4% ** | 11.4 ± 0.2% ** | 7.2 ± 0.2% ** |
| DOTAP/E7 600 nmol | 22.1 ± 2.1% | 14.3 ± 1.3% | 9.5 ± 0.9% |
| LPD/E7 100 nmol | 26.8 ± 2.2% | 11.0 ± 0.1% | 7.4 ± 0.1% |

** p< 0.01, *** p< 0.001 (n = 10 per group)

In addition, a significant decrease in the Treg population ($CD4^+Foxp3^+$ and $CD4^+CD25^+Foxp3^+$) was found in this treatment group, which is correlated with the results of anti-tumor activity. Similar results were observed in the LPD/E7 treated group. On the other hand, spleen cells collected from mice that received overdosed DOTAP did not show significant difference in the Treg population compared to the untreated tumor bearing mice. This result indicates that there is evidence for preferential expansion of T effector cells relative to Tregs after the treatment of DOTAP/E7 vaccine at an optimal lipid dose.

### 2.3.6 *In vitro* CTL-mediated and NK cell-mediated cytotoxicity

Since cytotoxic T lymphocytes (CTL) are most capable of killing tumor cells, vaccines that induce these immune responses are important for eradicating tumor growth or preventing cancer recurrences. To evaluate whether vaccination by DOTAP/E7 would be suitable for inducing a

primary CTL response, we prepared effector cells by collecting spleen cells from immunized mice at 1 week after the last immunization, followed by *in vitro* culture with 5 μg/ml E7 peptide for 5 days. As depicted in **Fig. 2.4a**, a cytotoxicity assay was carried out by incubating effector cells with PKH-67 labeled TC-1 target cells for 4 h and the percentage of killing was revealed by adding propidium iodide to label the killed cells, followed by flow cytometric analysis.

Spleen cells recovered from mice that received DOTAP/E7 at an optimal lipid dose exhibited significant CTL activity against TC-1 cells, while mice that received LPD/E7 or DOTAP overdose showed a moderate killing effect (**Fig. 2.4a**). We confirmed that the killing effect was E7-specific by incubating effector cells with E7-negative BL6 cells as targets, and negligible cytotoxicity was found. In addition to CTL-mediated killing, we also investigated natural killer (NK) cells-mediated cytotoxicity (**Fig. 2.4b**). NK cells are reported as being able to destroy tumor cells without deliberate immunization or activation and also play an important role in the innate immune response to interact with dendritic cells [27], [28]. In comparison with the PBS control, spleen cells directly harvested from mice that received liposomal peptide vaccines, without *in vitro* restimulation process, exhibited significant killing of YAC-1 cells, an NK susceptible target. The data indicated that DOTAP/E7 formulations were capable of stimulating NK cells, which were present in the effector cell population.

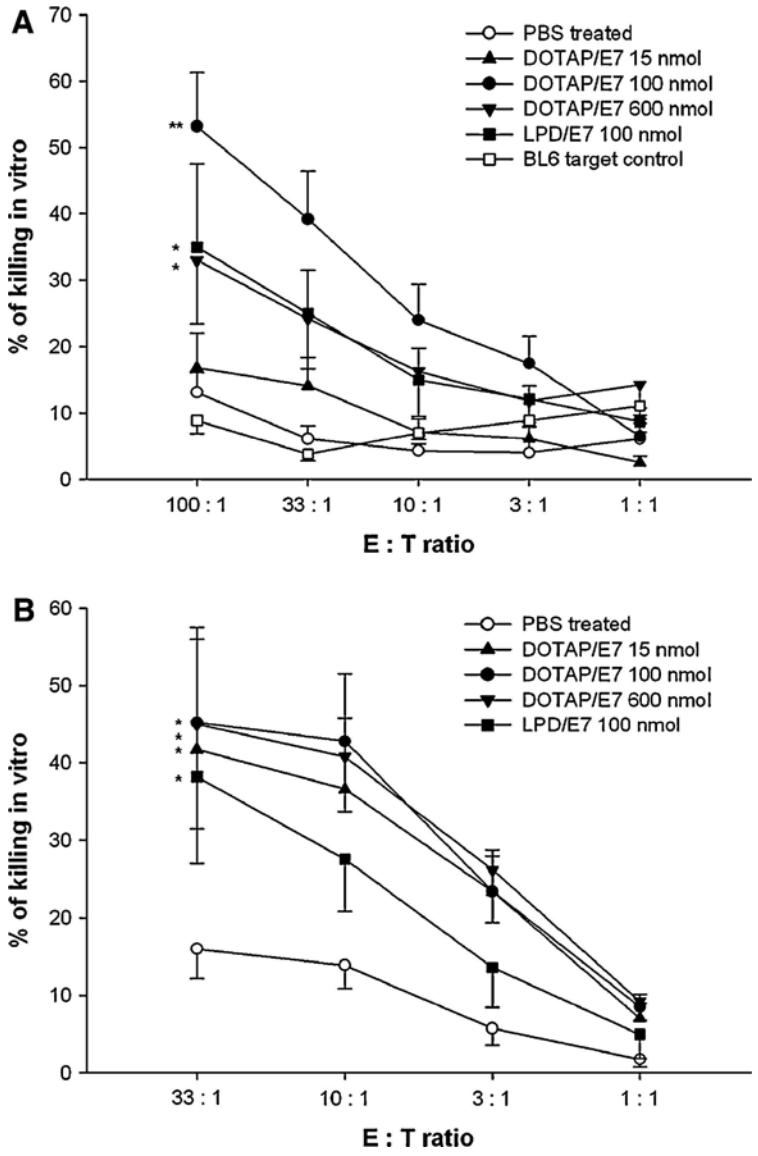


**Figure 2.4. Analysis for CTL-medicated and NK-mediated cytotoxicity by flow cytometry.**
A, E7-specific CTL clones were prepared and expanded as described. After 5 days *in vitro* restimulation with E7 peptide, effector cells were incubated with PKH-67 labeled TC-1 target cells at the indicated effector:target ratio for 4 h at 37°C. BL-6 was used as a non-specific target cell control. Percentage of E7-specific killing was determined by the proportion of PI positive cells within the gating of PKH-67 positive target cells. Statistical analysis was calculated by comparing treatment group and control group at 100:1 E:T ratio (*P <0.05, **P < 0.01, n =5). B, On day 7 after the last immunization, splenocytes from each group were harvested and incubated with PKH-67 labeled YAC-1 cells at the indicated E:T ratio for 4 h at 37°C. Percentage of killing was determined as described. Statistical analysis was done by comparing the treatment group and control group at 33:1 E:T ratio (*P <0.05, n =3).

### 2.3.7 $CD8^+$ T cells elicited by DOTAP/E7 are functionally active *in vivo*

*In vitro* CTL was measured after expansion of the splenocytes with antigen *in vitro*; however, there is a possibility of over- or underestimating the CTL function due to the restimulation process. Thus, we evaluated whether the observed *in vitro* CTL was also relevant *in vivo* (**Fig. 2.5a**). A mixture containing equal amounts of E7-pulsed $CFSE^{high}$ and unpulsed $CFSE^{low}$ spleen cells from a syngenic donor was injected iv into the mice, 1 week after the last DOTAP/E7 immunization. The specific lysis of target cells was analyzed by flow cytometry at 16 h after adoptive transfer. The percentage of E7-specific killing from mice that received an optimal dose of DOTAP/E7 or LPD/E7 was more than 65%, while in the groups that received DOTAP at low dose and overdose showed only 15 and 25% specific killing, respectively (**Fig 2.5b**).

IFN-γ secreted by activated T cells or NK cells play an important role in Th1 type immune response as well as inducing CTL response [29]. To assess whether the functional $CD8^+$ T lymphocytes induced by DOTAP/E7 vaccination would be able to produce the essential cytokine, spleen cells from control or immunized mice were isolated at 1 week after the final immunization and incubated with 5 μg/ml E7 peptide (**Fig. 2.5c**, lower panel) or without peptide (**Fig. 2.5c**, upper panel) for 6 h followed by intracellular staining of IFN-γ. The numbers represent the percentage of CD8 and IFN-γ double positive cells within the $CD8^+$ population. As depicted in **Fig. 2.5d**, the numbers of IFN-γ producing $CD8^+$ cells were significantly higher in mice that received E7 formulated in an optimal lipid dose of DOTAP and LPD than the control mice. The IFN-γ production by the $CD8^+$ cells was in an E7-specific manner. These results show that DOTAP at an optimal dose is a potent vaccine adjuvant for the induction of CTLs as well as generation of IFN-γ producing $CD8^+$ T lymphocytes in the systemic lymphoid organ.

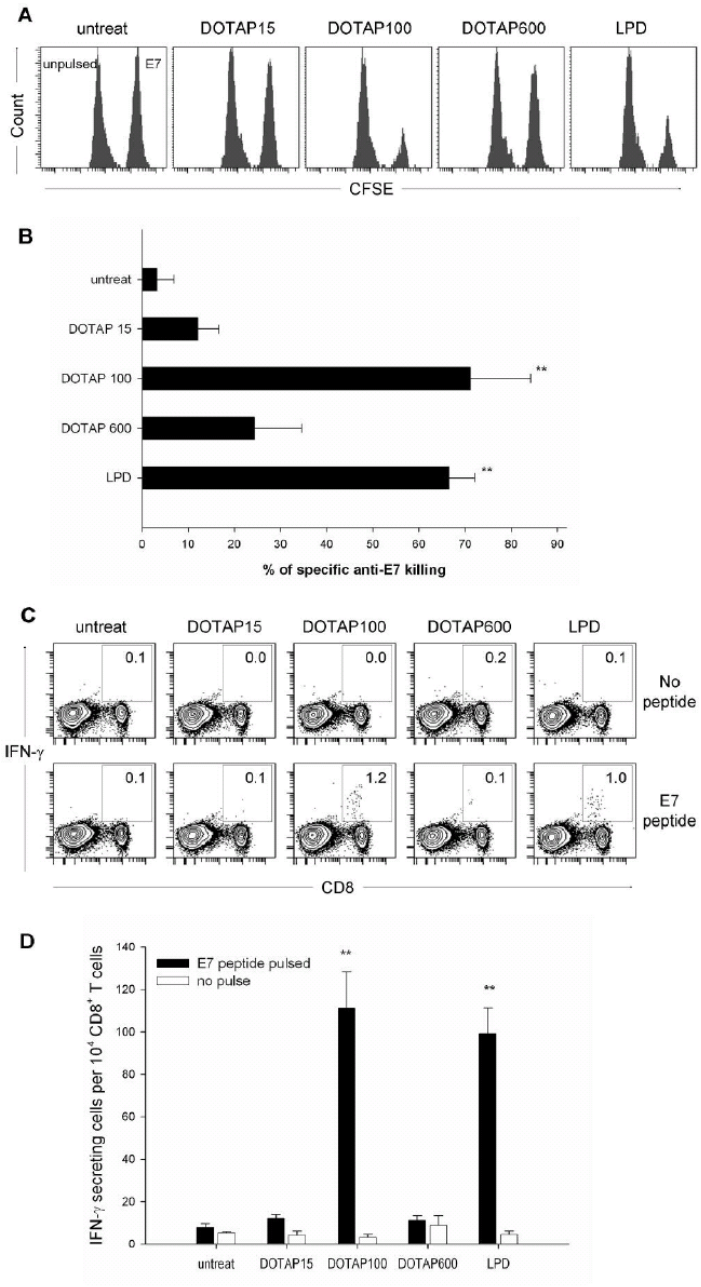


**Figure 2.5. Immunization with E7 peptide formulated in DOTAP adjuvant elicits functional $CD8^+$ T cells.**
A, C57BL/6 mice were immunized sc with 10 μg E7 peptide formulated in DOTAP at low, optimal, overdose or in LPD. Seven days after the last immunization, representative mice were injected iv with an equivalent amount of E7-pulsed (labeled with 4 μM CFSE) and non-pulsed (labeled with 0.4 μM CFSE) spleen cells from a syngenic donor. The spleen cells were harvested from the recipient mice 16 h later and the proportion of the $CFSE^{high}$ and $CFSE^{low}$ cells were analyzed by flow cytometry. B, Percentage of specific anti-E7 killing was compared to that of the untreated control (n = 4) and was statistically analyzed by t-test ($P < 0.01$). C, IFN-γ production by $CD8^+$ cells after *in vitro* stimulation with E7 peptide. The total spleen cells from immunized cells were stimulated with 5 μg/ml E7 peptide for 6 h, followed by subsequent staining with surface CD8 marker and intracellular IFN-γ prior to FACS analysis. The numbers shown on contour plots represent the percentage of $CD8^+IFN\gamma^+$ T cells gated on total $CD8^+$ cells. Representative figures in the four experiments performed. D, The numbers of $CD8^+IFN\text{-}\gamma^+$ cells per $10^4$ $CD8^+$ T cells were shown as mean ± SD and were compared to that of the untreated control (n =4, **$P < 0.01$).

### 2.3.8 ROS production induced by DOTAP liposome

Reactive oxygen species (ROS), which has been regarded as a harmful signal, plays an important role in DC activation as well as antigen presentation to the T cells [30]. Our results demonstrated that DOTAP/E7 complexes were mainly taken up by DC in the DLN, and high dose DOTAP dramatically decreased the antigen-specific T cell responses. Since high levels of ROS generated in cells lead to cell death [31], we hypothesized that a high dose of DOTAP lipids may lead to massive ROS production in DC in the DLN, resulting in cell death of DC. To test out the hypothesis, we measured ROS levels induced by DOTAP/E7 in the DLNs at 2 h after sc injection. For characterization of DC in the DLN, large granular cells were gated and analyzed by flow cytometry. In **Fig. 2.6a**, cells from mice that received DOTAP 15 nmol exhibited a basal level of ROS production (<5%), whereas cells from mice injected with DOTAP at an optimal dose produced relatively higher levels of ROS (20%). Strikingly, a majority of large granular cells (80%) from the DOTAP 600 nmol group showed positive ROS signal.

The subsequent question was whether the raised ROS induced by a high dose of DOTAP lipid triggered DC toward cell death. At 10 h post sc injection, DLNs were harvested and analyzed for cell death by flow cytometry. The percentage of cell death (propidium iodide positive) within the $CD11c^+$ population showed positive correlation with the lipid dose as well as with the level of ROS production shown in **Fig. 2.6b**. The percentage of cell death in the DC population was twice as high for the group injected with DOTAP overdose compared to the group of untreated control. Also shown in **Fig. 2.6b** are the numbers of live $CD11c^+$ cells per $10^5$ lymph node cells as a function of the dose of DOTAP lipid. The mice that received DOTAP/E7 with lipid 100 nmol exhibited the highest amount ($P < 0.01$) of live DC among all treatment groups. Indeed, the DLN weighed larger at 2 days after the mice received the optimal

formulation than other groups. Collectively, the results indicate that ROS toxicity induced by high dose DOTAP may cause cell death in DC.

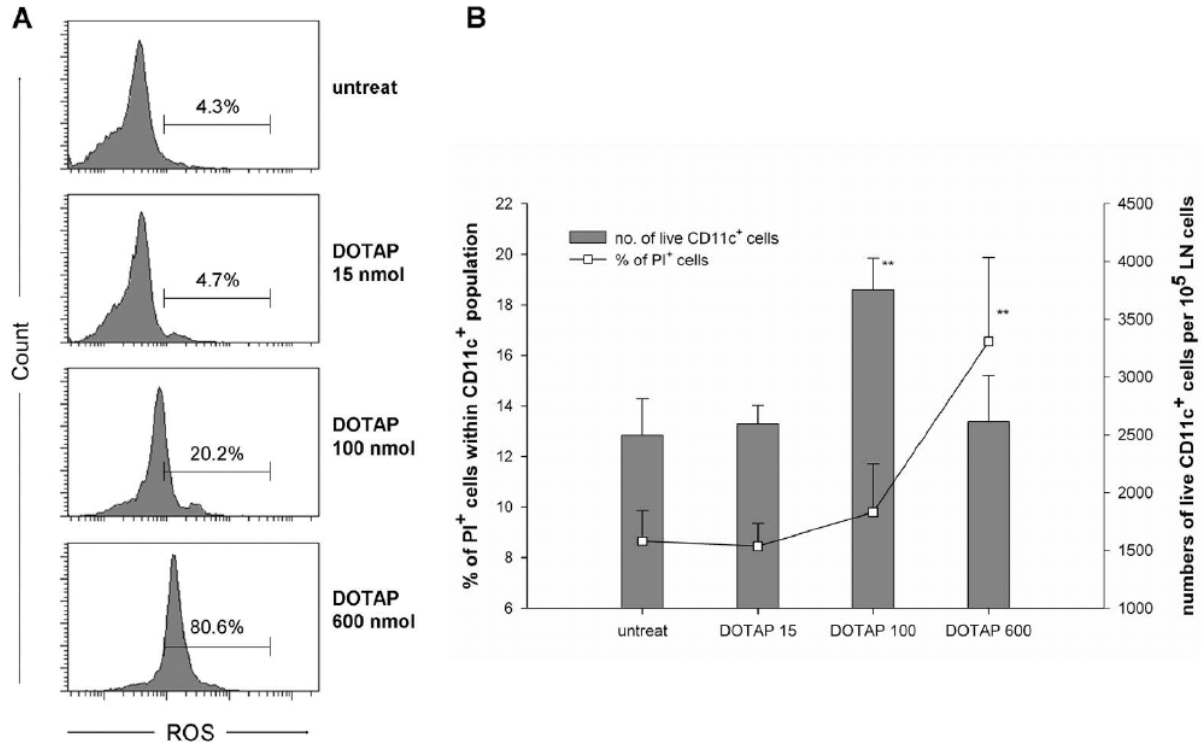


**Figure 2.6. High dose of cationic DOTAP liposome induces ROS production, resulting in cell death in DC.** A, DLNs from mice (n = 4) that received injection sc of DOTAP/E7 containing 0, 15, 100 or 600 nmol lipid were isolated 2 h after the injection. The total lymph node cells were incubated with DCFH-DA at 37°C for 30 min prior to the flow cytometric analysis. Large granular cells were gated and analyzed for the expression of ROS, where the fluorescent product DCF was generated in the presence of ROS. The relative percentages of cells with positive ROS signals are listed. B, Cytotoxicity in the DLN was measured by collecting cells at 10 h after DOTAP/E7 injection by flow cytometry. The relative percentages of dead cells ($PI^+$) in DC (open square) and the numbers of live DC per $10^5$ LN cells (bars) were shown and compared with that of the untreated control by paired Student's t test (**P <0.01).

Since DOTAP-induced ROS signal initiates the vaccine mechanism owing to the interaction of DC and the lipid, we found that the anti-cancer activity can be diminished by co-formulation of an inert lipid, DOPC with DOTAP/E7. As shown in **Fig. 2.7**, DOTAP at the optimal dose could induce the maximal vaccine activity and the desirable ROS content. However, both activities were significantly reduced when the same amount of DOTAP was diluted with an

inert neutral lipid, DOPC (1:5 dilution). In other words, the charge density of the cationic lipid could be an important parameter to ROS generation and the anti-cancer activity of the vaccine could be abolished by addition of an inert lipid.

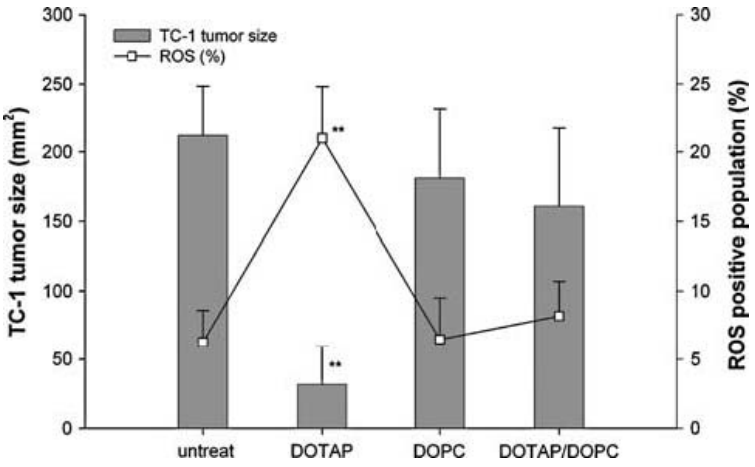


**Figure 2.7. ROS production in DLN was diminished by co-formulation of an inert neutral lipid, resulting in decreased anti-tumor activity of the vaccine.**
ROS production in DLN was measured 2 h after receiving sc DOTAP/E7, DOPC/E7 or DOTAP/DOPC/E7 (mole ratio of DOTAP/ DOPC = 1/ 5) formulation as described. Large granular cells were gated and the relative percentage of ROS positive population are shown (open square). TC-1 tumor bearing mice were treated with the corresponding vaccine formulation on day 6 and tumor sizes (bars) on day 23 were measured and compared between each group. **P < 0.01 was found for the comparison between DOTAP/E7 and DOTAP/DOPC group by paired Student's t test (n = 4-6).

## 2.4 DISCUSSION

As shown in the data of this report, we have demonstrated that the majority of the adjuvant activity of LPD comes from the DOTAP liposome and, for the first time, we have shown that cationic lipid alone is sufficient for a cancer vaccine adjuvant. Indeed, upon receiving the optimal lipid dose of DOTAP/E7, the particles were mainly taken up by DC, the major professional antigen presenting cells. The initiation of DC activation and migration to the DLN

facilitates immune responses against antigen-specific TC-1 tumor. Functional $CD8^+$ T lymphocytes were generated upon receiving DOTAP/E7 vaccine. The size of the tumor decreased and the tumor exhibited enhanced apoptosis, likely owing to the increasing number of infiltrating T cells in the tumor microenvironment. According to the bell-shaped dose response curve in **Fig. 2.1c**, the rising arc is very steep, indicating that DOTAP as a vaccine adjuvant is so potent that $EC_{50}$ is as low as about 15 nmol per injection. Herein, we have demonstrated that DOTAP/E7 formulation is truly a simple, safe, yet very efficient, therapeutic vaccine against pre-existing HPV positive tumor.

The E7 peptide RAHYNIVTF (amino acid 49-57) is positively charged and somewhat hydrophobic. The association of the peptide with the cationic liposomes must be due to hydrophobic interaction, because the percent encapsulation far exceeded what could be expected from the internal volume of the liposomes [32]. The suboptimal anti-cancer effect observed when low dose of lipids were used (i.e., 15 nmol DOTAP per dose) might be due to the insufficient number of vehicles to carry the required antigen to the lymphoid organs.

Merck's HPV vaccine, GARDASIL™, which is a preventive vaccine against HPV infection, is already available in the market. It contains HPV virus-like particles (VLPs), which mimic infectious virions in structure and morphology [33], [34] and potently induce high titered neutralizing antisera, even in the absence of any adjuvant [35], [36]. However, VLP vaccine is not likely to be active in treating established cervical cancer because the HPV coat proteins, which are major components of VLP, are not expressed in the cervical cancer cells. Our vaccine contains an epitope peptide of E7, which is expressed in the transformed cervical cells and can thus induce therapeutic immunity.

A great variety of experimental adjuvants are available for use in animals and some of them have been tested in clinical trials. They include several water-in-oil emulsions, liposomes and other chemical adjuvants [37]. However, only influenza virosomes [38] and Chiron's MF59 [39] have already been launched into the market, in addition to aluminum salts. Similar to cationic liposome, the submicron emulsion-based adjuvant, MF59, is internalized by dendritic cells [40]. It stimulates a variety of immune activities that lead to high antibody and T-cell reactions against co-delivered antigens. However, according to the clinical trial report on HSV and influenza vaccines [41], [42], evidence from animal models suggests that the MF59 adjuvant enhances neutralizing antibodies rather than T-cell responses [43]. Therefore, DOTAP liposome as a vaccine adjuvant is different from MF59 in that it generates a strong cell-mediated immune response, as shown by our data. We have compared the adjuvant activity of DOTAP with those of two other well-known adjuvants, i.e., CpG ODN and CFA. As shown in **Fig. 2.1d**, DOTAP compares favorably with them.

Some recently developed adjuvants such as MPL [44] and CpG ODN [12] were meant to initiate innate immunity by triggering toll-like receptors (TLR) on dendritic cells and macrophages, thus inducing the production of NF-κB and inflammatory responses. We have previously demonstrated that DOTAP cationic lipid was unable to enhance the expression of NF-κB, suggesting that stimulation of dendritic cells by cationic lipids is signaled through an NF-κB independent mechanism [16]. Thus, cationic liposomes could belong to a unique class of adjuvants with an improved safety profile.

DOTAP/E7, the therapeutic cancer vaccine described in this report, contains only two molecules, i.e., an antigen and a carrier. In addition to the delivery of the E7 peptide to the cytoplasm of the APC such DC, DOTAP must also lead DC to activation and maturation. To

elucidate the mechanism of DOTAP as vaccine adjuvant, we have demonstrated that the ability of cationic lipids to activate DC depends on the structure of the cationic lipid. Cationic lipids with a quaternary ammonium group are more potent than those with a tertiary ammonium group [18]. More recently, we have reported that DOTAP and other active cationic lipids can activate MAP kinase ERK and p38 pathway [19] in DC, resulting in chemokines and cytokines production, especially CCL2. All of the preceding results support our hypothesis that DC plays an important role in response to DOTAP stimulation.

The ROS has been implicated in innate immune response and T cell activation [30], [45], and excess of ROS production leads to cell death [46], [47]. We have shown in this report that ROS was induced by DOTAP in cells in the DLN and a high dose of DOTAP lipid led to killing of DC. Indeed, Iwaoka et al. have shown that cationic liposomes can induce ROS in macrophages *in vitro* [31]. Data shown in **Fig. 2.6a** clearly demonstrate that DOTAP was capable of generating ROS signal *in vivo* in the DLN after sc injection of DOTAP/E7 complexes. The same data also suggest that excess ROS generated by a high dose of DOTAP leads to enhanced death of DC. There might be other possible reasons related to the loss of anti-cancer activity by overdose of DOTAP/E7; however, the decreased amount of activated APC in the DLN should definitely play an important role in the observed decrease in the lymphocyte infiltration (**Fig. 2.3**), antigen-specific CTL activities and IFN-γ production (**Figs. 2.4** and **2.5**) and, most importantly, the anti-tumor activity (**Fig. 2.1**) at the high vaccine dose. On the other hand, a desirable level of ROS production is required since DOTAP-induced ROS is probably the initial signal to mediate the subsequent signal transduction, such as the ERK and p38 MAP kinases, necessary for the vaccine activity [19]. We have demonstrated the importance of the density of the cationic lipid to the anti-cancer activity in **Fig. 2.7** where we showed that both

ROS generation and anticancer activity could be diminished by co-formulation of an inert, neutral lipid, DOPC with DOTAP/E7. The result indicates that the simple cationic DOTAP/E7 formulation is not just a sufficient, but also a necessary, vaccine formulation for the treatment of cancer.

A high dose of DOTAP seems capable of stimulating CTL, in **Fig. 2.4a**, using a traditional assay method, which requires *in vitro* stimulation of responder T cells with the antigen for 5 days. We cannot rule out the possibility that the true functionality of $CD8^+$ cells was overestimated after *in vitro* expansion of the subset cells. An *in vivo* CTL result (**Fig. 2.5a**) gave us a better estimation of the functionality of $CD8^+$ T cells elicited in the mice and the data showed that a high dose of DOTAP does not generate sufficient CTL *in vivo*. Although DOTAP at high dose could also generate a good NK response toward non-specific killing (**Fig. 2.4b**), the effect contributed little to the anti-tumor activity. It is not clear, at present, the role that NK cells play in the overall anti-tumor activity induced by DOTAP/E7. We have previously shown that the IFN-γ activity in the splenocytes of mice treated with LPD/E7 was independent of NK, since mice injected with NK1.1 antibody to eliminate the NK activity showed unchanged IFN-γ activity (Dileo et al., unpublished data). Mice injected with DOTAP alone without E7 did not show significant anti-tumor activity (**Fig. 2.1b**), suggesting that the antigen independent component was minor.

T regulatory cells (Tregs) were initially described by Gershon et al. [48, 49] in the 1970s and were called suppressive T cells. Recent studies have explored the role of $CD4^+CD25^+$ Tregs in the suppression of tumor immunity in several murine models as well as in cancer patients [50]. The frequency of Treg population increases in the peripheral blood of cancer patients [51]. They are also enriched among the tumor infiltrating lymphocytes and in the DLNs [52]. Also,

accumulation of Treg in tumor-associated tissue predicts poor prognosis or survival [5]. Although the detailed mechanism of how Treg dampens normal T-cell immunity is not well understood, it has been reported that the anti-tumor activity is enhanced by using anti-CD25 antibody to block Treg cells [53]. Indeed, it has become apparent that it will be necessary to monitor and characterize both the effector and Treg responses in patients that receive candidate human tumor vaccines [5]. In the present studies, we found a correlation of the anti-tumor activity of the DOTAP/E7 (**Fig. 2.1b**) with the reduction of Treg cells (**Table 2.1**). It was evident that the reduced anti-tumor activity of DOTAP/E7 at a high dose was associated with a higher level of Tregs, probably owing to the excess ROS production and cell death in DC. However, we do not have direct evidence to show that a high dose of DOTAP increases Tregs subpopulation.

In summary, the promising features of the DOTAP/E7 formulation, which contains only two molecules, include the safety and stability of the complexes and the abilities to elicit DC activation, generate antigen-specific CTL, as well as to inhibit an established tumor after a single dose of immunization. It also lends itself to the mechanistic studies of how DC can be activated by cationic lipids and how liposomes deliver the peptide antigen to DC. Although the dose of cationic DOTAP lipid as a vaccine adjuvant should be carefully studied before going to the clinical trial, it possesses great potentials that deserve further studies.

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# 3.0 INDUCTION OF CYTOTOXIC T-LYMPHOCYTES AND ANTI-TUMOR ACTIVITY BY A LIPOSOMAL LIPOPEPTIDE VACCINE

We have previously described a simple yet effective liposome-based therapeutic vaccine, DOTAP/E7, which contains only two molecules, the cationic lipid DOTAP and a peptide antigen derived from the E7 oncoprotein of human papillomavirus (HPV) type 16. In the current report, we have improved the vaccine formulation by incorporation of E7-lipopeptide instead of the water-soluble native E7 peptide into the DOTAP liposome. The lipopeptide consists of an N-terminal α or ε-palmitoyl lysine connected to the E7 peptide via a dipeptide Ser-Ser linker. The DOTAP/E7-lipopeptide vaccine exhibited an enhanced functional antigen-specific $CD8^+$ T lymphocyte response *in vivo* compared to the previous DOTAP/E7 formulation. More importantly, the cytotoxic T cells induced by the DOTAP/E7-lipopeptide vaccine could efficiently eliminate an existing HPV positive TC-1 tumor. The anti-tumor activity of lipopeptide formulated in DOTAP liposome was more than twice as that of native E7, likely owing to the increased peptide entrapment efficiency in the liposomal complex. Our results also showed that it is essential to have the dipeptide spacer sequence between E7 peptide and the attached fatty acid to achieve a full immune response. Overall, the improved DOTAP/E7-lipopeptide vaccine described herein showed a significantly enhanced therapeutic effect for the treatment of a cervical cancer model.

## 3.1 INTRODUCTION

Liposomes have been extensively used for delivering small molecular weight drugs [1], plasmid DNA [2], oligonucleotides [3], proteins [4, 5] and peptides [6]. From a safety standpoint, a liposomal vehicle as a non-viral vaccine carrier has been regarded as a preferable strategy compared to traditional immunizations using live attenuated vaccines or viral vectors such as vaccinia or influenza virus [7, 8]. Recent studies from our group have led to the development of a simple yet effective peptide-based liposomal vaccine, DOTAP/E7 complex, which consists only two molecules, the cationic lipid DOTAP and a peptide antigen which is derived from E7 oncoprotein of human papillomavirus (HPV) type 16. The DOTAP/E7 formulation was able to induce both preventative and therapeutic anti-tumor effects against HPV positive TC-1 tumor in a mouse model. Our results have demonstrated for the first time that the cationic liposome alone not only plays the role of a carrier to deliver the peptide antigen to the antigen presenting cells (APC), but also serves as a potent vaccine adjuvant to stimulate immune responses and initiate DC-T cell interactions [9, 10].

There has been considerable interest in developing lipopeptide formulations for both humoral and cellular immune response. This is in contrast to the native synthetic peptides which are poorly immunogenic. Peptides linked with mono- or multi- palmitic acid are capable of producing antigen specific cytotoxic T lymphocyte response against infectious diseases such as HIV [11, 12], HBV [13] and malaria [14] and several clinical trials are investigating this effect [15, 16]. However, lipopeptides have limited solubility and require a means for solubilization. Since liposome is a good carrier for lipophilic drugs, we investigated the possibility of formulating a lipopeptide antigen in the DOTAP liposomes. Our results showed significantly improved immunogenic activity of the lipopeptide as compared to the original E7 peptide. We

have also evaluated the anti-tumor activity of the E7-lipopeptide in DOTAP liposome in the TC-1 cervical cancer model in mice.

## 3.2 MATERIALS AND METHODS

### 3.2.1 Lipids, reagents and murine tumor cell lines

DOTAP was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Murine TC-1 cells were kindly provided by Dr. T.C. Wu at Johns Hopkins University (Baltimore, MD). TC-1 cells are C57BL/6 mouse lung epithelial cells transformed with HPV 16 E6 and E7 oncogenes and the activated H-ras. RMA-S, a mouse lymphoma cell line, was kindly provided by Dr. J. Frelinger at University of North Carolina at Chapel Hill. Both H-$2^b$ murine tumor cell lines were maintained in RPMI-1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin (Invitrogen).

### 3.2.2 Peptide Synthesis

Peptides and lipopeptides (**Table 3.1**) containing an H-$2D^b$ restricted CTL epitope (amino acid 49-57, RAHYNIVTF) derived from HPV 16 E7 protein were synthesized and purified in Molecular Medicine Institute Peptide Synthesis Facility in the University of Pittsburgh. Briefly, lipopeptides used in this study were generated by elongating the N-terminal of E7 peptide with a linker peptide Lys-Ser-Ser (KSS) and a palmitic acid was then attached to the alpha or epsilon amino group of the Lys residue. An unlipidated version of KSS-elongated E7 peptide

(abbreviated KSS-E7) or a palmitoylated E7 peptide (fatty acid attached to N-terminal Arg residue without a spacer sequence; abbreviated PA-E7) were used as controls.

### 3.2.3 Preparation and characterization of DOTAP/lipopeptides

Cell culture grade water (Cambrex, Walkersville, MD) was used in all liposome preparations. Lipopeptide was mixed with DOTAP in $CHCl_3$/MeOH (1:1 mixture) followed by evaporation under a steady stream of dry nitrogen gas. Traces of organic solvent were removed by vacuum desiccation overnight. Lipopeptide/lipid films were hydrated by adding water and incubated at room temperature for 12 h. The suspensions were sonicated in a bath type sonicator for 10 min followed by extrusion (Hamilton Co., Reno, NV) through 400, 200 and 100 nm polycarbonate membrane (Nuclepore, Pleasanton, CA), and were stored at 4ºC before use. For preparation of DOTAP/E7 or DOTAP/KSS-E7, DOTAP lipid film was hydrated in an aqueous solution containing the water-soluble peptide.

Peptide encapsulation efficiency was determined by the percentage of the liposome-bound peptide using LavaPep™ peptide quantification kit (Fluorotechnics, Sydney, Australia). Since unincorporated lipopeptide aggregated and could not pass the exclusion filter, the incorporated lipopeptide was measured as the amount associated with the extruded liposomes. The liposomes were dissolved in 1% SDS and the amount of peptide was measured as described above. The data were reported as the mean ± SD (n=3). For water-soluble peptide such as native E7 and KSS-E7, the unbound peptide was separated from the complex by a Microcon® centrifugal filtration device (Millipore, Bedford, MA) [9]. The concentration of the unbound peptide was measured using LavaPep™ according to the manufacturer's instructions. The

efficiency of encapsulation was determined as (100 - % unbound peptide) and was reported as the mean ± SD (n=3).

### 3.2.4 Mice and immunizations

All work performed on animals was in accordance with and approved by our institutional IACUC. C57BL/6 female mice of 6-7 weeks old were purchased from Charles River Laboratories (Wilmington, MA) and were used in all animal studies. Subcutaneous tumors were established by injecting TC-1 cells ($10^5$ cells) into the hair-trimmed flank of the mouse on day 0. Mice were treated with a liposomal formulation subcutaneously (s.c.) at the other side of flank on day 6 unless otherwise stated. The size of the tumor was measured using a caliper two or three times a week. Tumor size was determined by multiplying the two largest dimensions of the tumor.

### 3.2.5 Analysis of *in vivo* CTL response

*In vivo* CTL activity of E7-specific cytotoxic T cells was enumerated according to the protocol of Byers et al. [17] with minor modifications. In brief, spleen cells from syngenic mice were RBC lysed followed by pulsing with 10 μM E7 peptide or without peptide in complete medium for 1 h at 37°C. Both spleen cell populations were stained with equal amount of 2 μM PKH-26 (Sigma-Aldrich) according to manufacturer's instruction. The peptide pulsed and unpulsed populations were loaded with 4 and 0.4 μM CFSE (Molecular Probes), respectively, at 37°C for 15 min. The two cell populations were mixed together (1:1) for tail vein injection to the control or the immunized mice ($10^7$ cells per mouse). At 16 h after injection, spleen cells from the

recipient mice were isolated and single cell suspension were prepared prior to flow cytometric analysis. The number of $CFSE^{high}$ and $CFSE^{low}$ population were determined and the *in vivo* E7 specific lysis percentage was enumerated according to a published equation [17].

### 3.2.6 Intracellular cytokine staining

All anti-mouse antibodies used for flow cytometric analysis were purchased from BD Pharmingen (San Diego, CA) or eBioscience, Inc (San Diego, CA). For the measurement of IFN-γ producing $CD8^+$ T cells, spleen cells were isolated from the control or the immunized mice 7-10 days after the last immunization. $2 \times 10^6$ spleen cells were incubated with 5 μg/ml E7 peptide or without peptide for 6 h in the presence of 1 μl/ml of GolgiPlug (BD Pharmingen). After washing by FACS buffer, cells were stained directly with anti-CD8a (53-6.7) on ice for 30 min. Prior to staining with anti-IFN-γ mAb (XMG1.2), cells were fixed and permeabilized using the Cytofix/Cytoperm™ kit (BD Pharmingen) according to the manufacturer's instruction. Cells were finally resuspended in 300 μl stain buffer and analyzed using a flow cytometer.

### 3.2.7 MHC class I binding assay

RMA-S cells at a concentration of $5 \times 10^5$ cells/ml were incubated overnight at 27°C with native E7 or KSS-E7 peptide (10 μM). Cells incubated with medium were used as a control. Cells were then transferred to 37°C and incubated for 2 h. After washes, cells were stained with fluorescently conjugated mAbs against $H\text{-}2D^b$ or $H\text{-}2K^b$ molecules on the cell surface prior to flow cytometry analysis.

### 3.2.8 Statistical analysis

Data were analyzed statistically using a two-tailed Student's t-test. Differences in data were considered statistically significant when p value was less than 0.05.

## 3.3 RESULTS

### 3.3.1 Encapsulation efficiency of E7-lipopeptide in the cationic DOTAP liposome

In order to improve the efficiency of the DOTAP/E7 therapeutic vaccine, one of the strategies was to increase the incorporation of the antigen in the liposome by utilizing lipopeptides. The lipopeptides used in this work are described in **Table 3.1**. When peptide loading was 2.5 mol% of the total lipid, the entrapment efficiency of E7-lipopeptides (i.e., α- or ε-PA-KSS-E7) within DOTAP liposome reached up to 90% compared to ~25% incorporation of the water-soluble native E7 and KSS-E7 peptides.

**Table 3.1. Composition of the synthetic lipopeptides and the peptide entrapment efficiency in the DOTAP liposome at molar ratio lipopeptide: DOTAP = 1:40.**

| Peptide name | Peptide composition | | | Entrapment efficiency (%) |
|---|---|---|---|---|
| | Lipid | Spacer seq. | CTL epitope | |
| Native E7 | - | - | RAHYNIVTF | 27.1 ± 5.8 % |
| KSS-E7 | - | KSS | RAHYNIVTF | 26.4 ± 6.1 % |
| PA-E7 | Palmitic acid | - | RAHYNIVTF | 84.5 ± 6.4 % |
| α-PA-KSS-E7 | Palmitic acid | KSS | RAHYNIVTF | 92.8 ± 4.2 % |
| ε-PA-KSS-E7 | Palmitic acid | KSS | RAHYNIVTF | 94.1 ± 4.6 % |

As shown in **Fig. 3.1**, varying amounts of ε-PA-KSS-E7 lipopeptide (0.16 mol% to 10 mol%) were dissolved with DOTAP lipid of 100 nmol in an organic solvent and the incorporation rate was measured following liposome preparation. For all lipopeptide complexes, the entrapment efficiency decreased with increasing lipopeptide loading. The incorporation rate was nearly 95% when lipopeptide concentration did not exceed 2.5 mol%. When the peptide loading was increased to 5 mol%, approximately 70-75% entrapment efficiency was measured. Furthermore, less than 60% of lipopeptide was incorporated into the liposomes when lipopeptide was loaded at 10 mol%. The lost lipopeptide at higher loading ratio could be mostly recovered from the polycarbonate membrane which was used during extrusion (data not shown). These results demonstrate that lipopeptides exhibit superior entrapment efficiency compared to the native E7 peptide. However, the incorporation rate decreases with the increasing loading of lipopeptide; it exceeded the solubility limit at the high loading ratios.

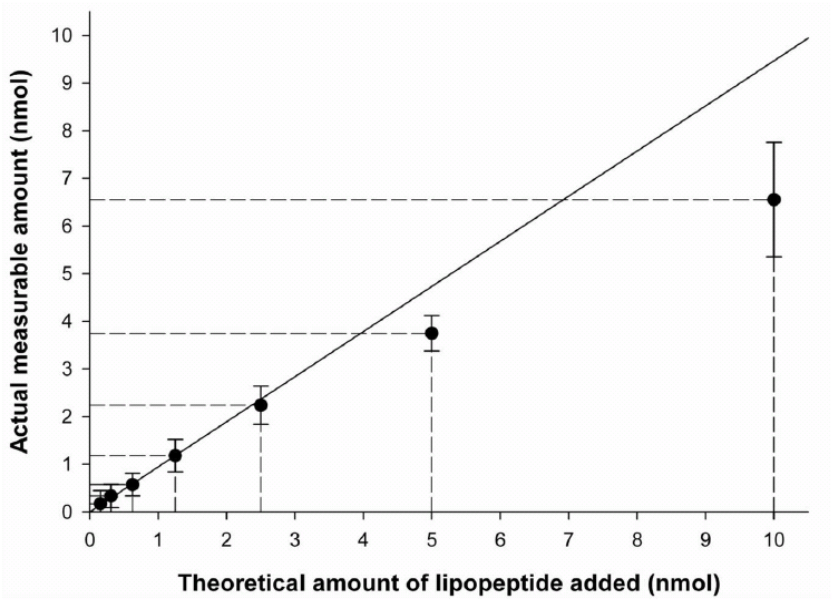


**Figure 3.1. The entrapment efficiency of the E7-lipopeptide in DOTAP liposome.**
Lipopeptide varying from 0.16 to 10 nmols was mixed with DOTAP lipid of 100 nmol and the peptide entrapment efficiency was measured after liposome preparation. The solid line shows a regression curve w/ a slope equal to 95%.

### 3.3.2 Anti-tumor activity was enhanced by incorporation of E7-lipopeptide in the DOTAP liposome

Previous studies in our group have shown that a MHC class I peptide epitope derived from HPV (type 16) E7 protein encapsulated in cationic DOTAP liposomes can generate a therapeutic effect against the HPV16 E7 positive tumor cell line, TC-1 [9, 10]. To test whether the anti-tumor activity is enhanced by use of the lipopeptide, a series of E7-lipopeptides (**Table 3.1**) were synthesized and formulated in the DOTAP liposomes as described.

As shown in **Fig. 3.2**, TC-1 tumor bearing mice were given a single-dose treatment on day 6 with DOTAP/E7 (containing 10 nmol or 5 nmol peptide) or DOTAP/E7-lipopeptide formulations containing 5 nmol of the antigen. DOTAP/E7 with 5 nmol of peptide (suboptimal dose) did not show a significant anti-tumor activity. On the contrary, both of the E7-lipopeptides (α- or ε-PA-KSS-E7) at 5 nmol of antigen, when formulated in DOTAP liposome, showed a significantly enhanced therapeutic effect (**$p < 0.01$) compared to the native E7 of 5 nmol. The anti-tumor activity elicited by the lipopeptides was similar to that of DOTAP/E7 at 10 nmol (i.e. optimal peptide dose). DOTAP liposome alone did not show anti-tumor activity. PA-E7, which is a palmitoylated E7 peptide (without the KSS spacer), when formulated in DOTAP liposome failed to show an enhanced anti-tumor activity as seen in other lipopeptide formulations, likely owing to the epitope being hidden by directly attaching a fatty acid to the peptide. In addition, an unlipidated version of KSS-elongated E7 peptide (KSS-E7) exhibited a larger effect (*$p < 0.05$) in tumor suppression than the native E7. Under these conditions, E7-lipopeptides formulated in DOTAP liposome showed an enhanced anti-tumor activity compared to the original DOTAP/E7 formulation.

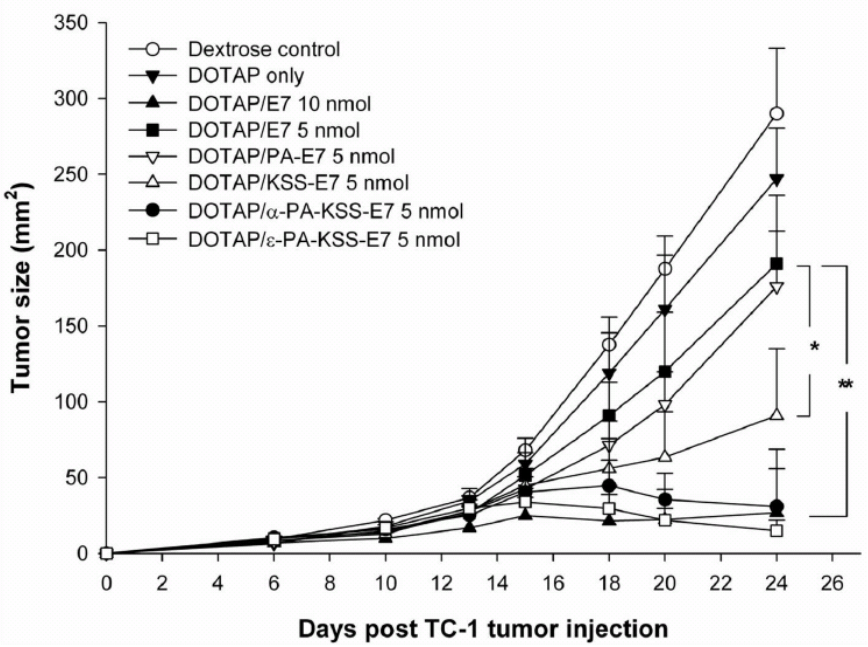


**Figure 3.2. Anti TC-1 tumor activity was enhanced by incorporation of E7-lipopeptide in the cationic liposomal formulation.**
TC-1 tumor bearing mice (8-12 mice per group) received a single treatment on day 6 with DOTAP/E7 (containing E7 peptide of 5 or 10 nmols) or DOTAP/E7-lipopeptide (containing 5 nmol peptide) or DOTAP lipid alone. Mice treated with dextrose (5 %) were used as a negative control. Tumor size was determined by multiplying the two largest dimensions of the solid tumor. The mean of tumor sizes of each group at day 24 was compared to that of the group which was received DOTAP/E7 (5 nmol) and was analyzed by Student's t-test (* $p < 0.05$, ** $p < 0.01$).

### 3.3.3 *In vivo* cytotoxic T-lymphocytes were elicited by DOTAP/E7-lipopeptide formulation

It is important to evaluate whether a candidate cancer vaccine would be suitable for inducing a primary cytotoxic T-lymphocyte (CTL) response since CTLs are essential for eradicating tumor growth and preventing cancer recurrences [18, 19]. To avoid the possible overestimation of CTL function due to *in vitro* restimulation and expansion process, we evaluated CTL induced *in vivo* by DOTAP/E7 or DOTAP/E7-lipopeptide vaccine (**Fig. 3.3a**).

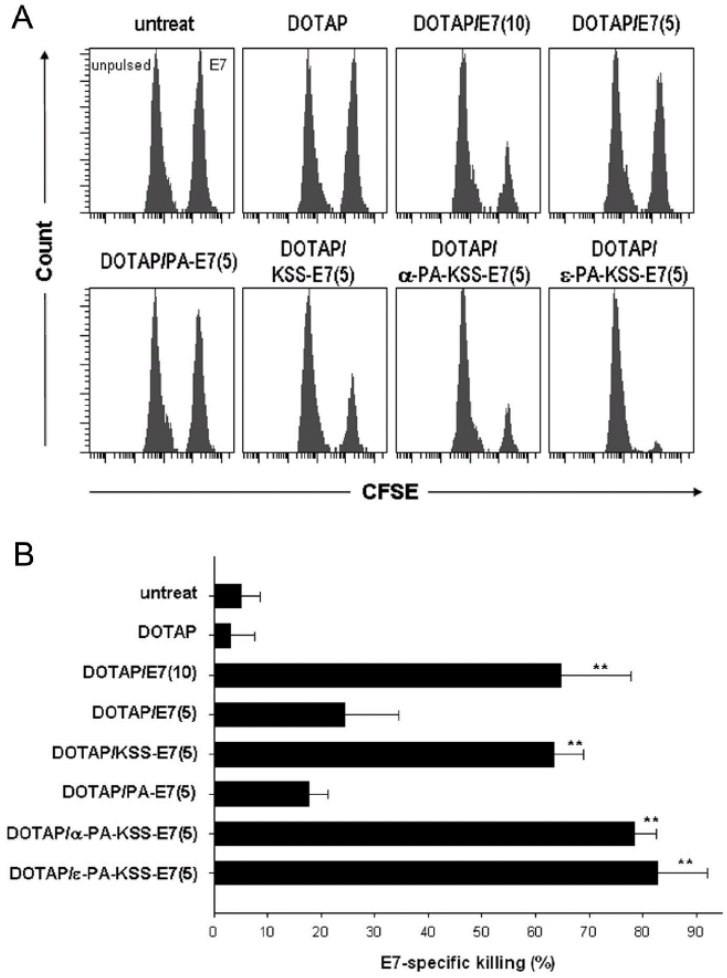


**Figure 3.3. Immunization w/ DOTAP/E7-lipopeptide formulation elicited an enhanced *in vivo* CTL response.** A, C57BL/6 mice were s.c. immunized with DOTAP/E7 (containing E7 peptide of 5 or 10 nmols), DOTAP/KSS-E7 (5 nmol), DOTAP/E7-lipopeptide (5 nmol) or DOTAP lipid alone at day 0 and day 10. Mice left untreated were used as a negative control. Seven days after the last immunization, representative mice were i.v. injected with an equivalent amount of E7-pulsed (labeled with 4 μM CFSE) and non-pulse (labeled with 0.4 μM CFSE) spleen cells obtained from a syngenic donor. 16 h later, spleen cells from the adoptively transferred mice were harvested and the proportions of the $CFSE^{high}$ and $CFSE^{low}$ cells were analyzed by flow cytometry. B, Percentage of specific anti-E7 killing was shown. The mean of the percentage from each group was compared to that of the group received DOTAP/E7 (5 nmol) and was statistically analyzed by t-test (** $p < 0.01$, $n = 4$).

As described in Materials and Methods, a mixture containing equal amounts of E7-pulsed $CFSE^{high}$ and unpulsed $CFSE^{low}$ spleen cells from a syngenic donor was i.v. injected into mice at 7 days after the last immunization. The specific lysis of E7-pulsed cells was analyzed by flow cytometry at 16 h after the adoptive transfer. The mice immunized with DOTAP/E7 antigen (10

nmol) could generate a superior E7-specific killing compared to those immunized with lower amounts of antigen DOTAP/E7 (5 nmol). In contrast to the native E7 formulation, mice receiving 5 nmol α- and ε-PA-KSS-E7 but not PA-E7 formulated in DOTAP liposome efficiently eliminated about 80% of E7-pulsed targets (**Fig. 3.3b**). In addition, the mice receiving DOTAP/KSS-E7, were also capable of killing 60% of *in vivo* targets while the mice receiving DOTAP alone did not generate a noticeable E7-specific CTL response. The results suggested that E7-lipopeptides formulated in DOTAP liposome elicited an improved *in vivo* CTL response in than the native E7 peptide.

### 3.3.4 IFN-γ production from functional $CD8^+$ T cells was induced by DOTAP/E7-lipopeptide

To assess the epitope-specific immune response induced by DOTAP/E7-lipopeptide vaccination, IFN-γ producing $CD8^+$ T cells were analyzed (**Fig. 3.4**). Spleen cells from control or immunized mice were isolated at one week after the final immunization. After stimulation with 5 μg/ml E7 peptide (**Fig. 3.4a**, lower) or without peptide (**Fig. 3.4a**, upper), intracellular staining for IFN-γ was performed. The numbers shown in the dot plots represent the percentage of CD8 and IFN-γ double positive cells within the $CD8^+$ population. As depicted in **Fig. 3.4b**, the numbers of IFN-γ producing $CD8^+$ cells were significantly higher in mice that received 5 nmol of ε-PA-KSS-E7 formulated in the DOTAP liposomes than those of both 10 and 5 nmols of the native E7 formulation (**$p < 0.01$). Again, KSS-E7 showed a superior result compared to the native E7 at the equal antigen amount. The IFN-γ production by the $CD8^+$ cells was in an E7-specific manner, as the unpulsed cells showed only background level of the cytokine. These results show that

incorporation of α- or ε-PA-KSS-E7 lipopeptides into DOTAP liposomes clearly enhanced the amount of IFN-γ producing $CD8^+$ T lymphocytes in the lymphoid organ.

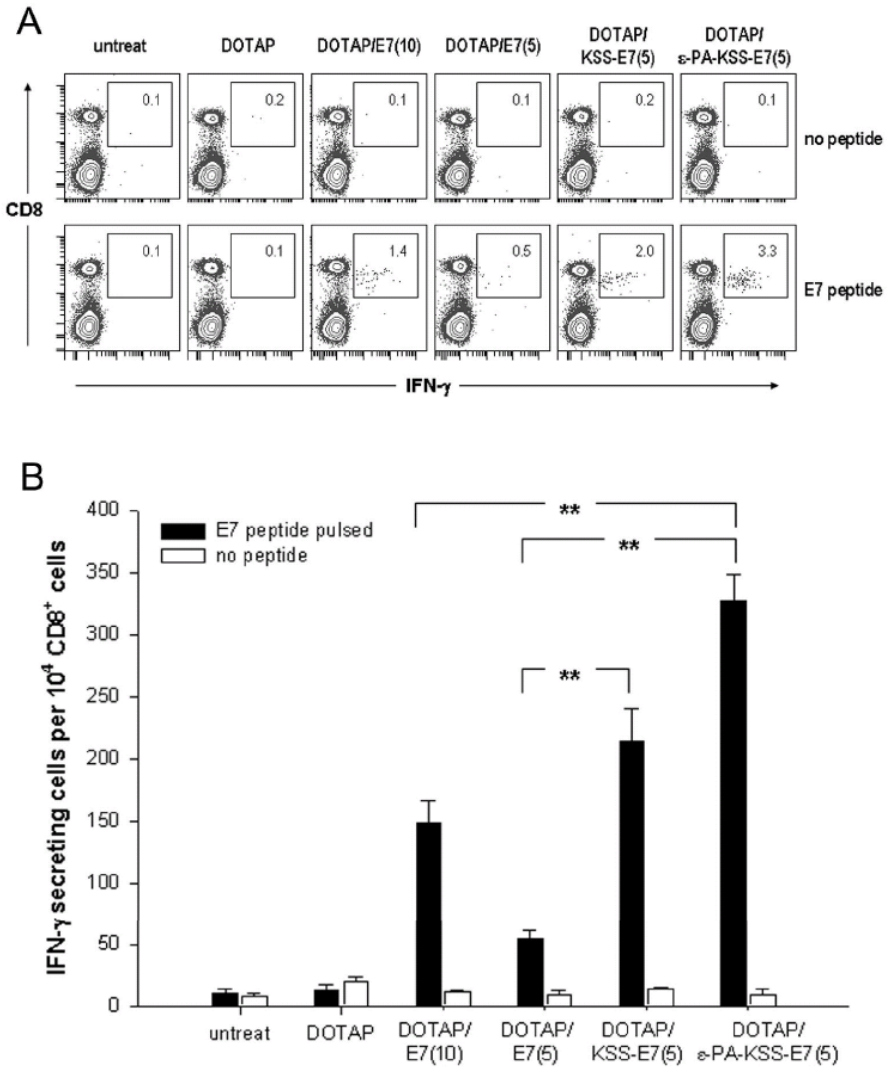


**Figure 3.4. Immunization w/ DOTAP/E7-lipopeptide increased the production of IFN-γ secreting $CD8^+$ T cells.** A, Mice were immunized as described and seven days after the last immunization, spleen cells from immunized mice were isolated. The cells were stimulated *in vitro* with or without E7 peptide (5 μg/ml) for 6 h, and were stained with a surface CD8 marker and an intracellular IFN-γ cytokine prior to FACS analysis. The numbers shown on contour plots represent the percentages of $CD8^+IFN\text{-}\gamma^+$ T cells gated on the total $CD8^+$ cells. Representative figures in four experiments performed. B, The percentage of $CD8^+IFN\text{-}\gamma^+$ double positive cells per $10^4$ total $CD8^+$ from each treatment group were shown as mean ± SD and were statistically analyzed by paired t-test (** $p < 0.01$, $n = 4$).

### 3.3.5 H-2D$^b$ was up-regulated by KSS-E7 using RMA-S binding assay

Both native E7 and KSS-E7 are water-soluble peptides that reach similar entrapment efficiency in liposome. KSS-E7 appears to have a better immunogenicity compare to the native E7. To elucidate the mechanism by which KSS-E7 peptide exhibited superior antigen activity than the native E7, the binding of MHC class I molecules by E7 and KSS-E7 peptide was investigated. E7 peptide (a.a. 49-57) is a known epitope restricted to H-2D$^b$ [20] and it up-regulated more than 4-fold of H-2D$^b$ molecules on RMA-S cells compared to the control (**Fig. 3.5**). Intriguingly, an 8-fold increase in the mean fluorescence was observed for KSS-E7 peptide. No up-regulation of H-2K$^b$ molecules was detected on RMA-S cells after incubating with either E7 or KSS-E7 peptide. The results suggest that KSS-E7 has an improved binding affinity for H-2D$^b$ molecules than native E7 peptide, which may lead to an overall superior anti-tumor activity when formulated in the DOTAP liposomes.

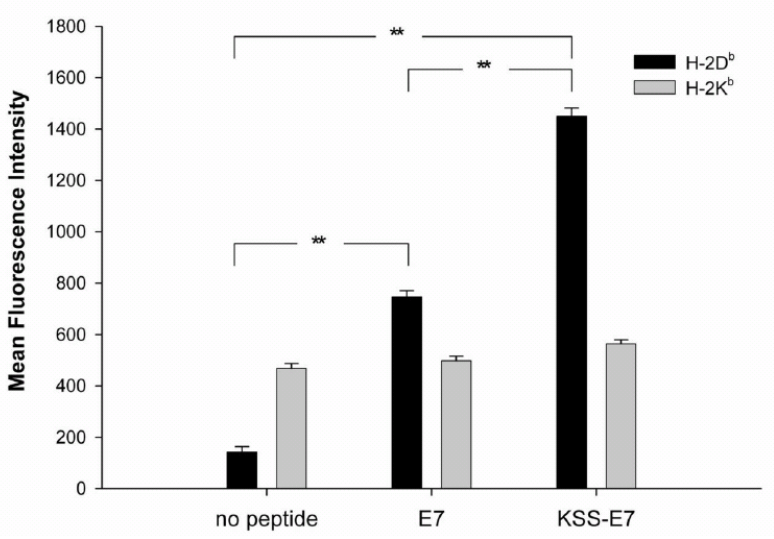


**Figure 3.5. MHC class I molecules up-regulation by E7 and KSS-E7 peptide.**
RMA-S cells were incubated with E7 or KSS-E7 peptide at 10 μM and the up-regulation of MHC class I molecules was detected by a fluorescently conjugated mAb against H-2D$^b$ or H-2K$^b$. Cells treated with medium were used as a control. The results are expressed as mean fluorescence intensity of triplicate determination ± SD and were statistically analyzed by paired t-test (** $p < 0.01$, $n = 3$)

### 3.3.6 Potent anti-tumor activity was induced by DOTAP/E7-lipopeptide vaccine

To further study the potency of the DOTAP/E7-lipopeptide vaccine against the existing tumor, the varied doses of ε-PA-KSS-E7 (i.e. 1.25 to 10 nmols per injection) were investigated (**Fig. 3.6a**). The tumor bearing mice that received one single treatment of DOTAP/ε-PA-KSS-E7 with 5 or 10 nmol of antigen could induce full tumor growth inhibition (** $p < 0.01$) compared to the partial tumor growth inhibition in those injected with DOTAP/E7 (5 nmol). The formulation still showed a partial anti-tumor effect (* $p < 0.05$) when the lipopeptide amount was decreased to as low as 1.25 nmol.

To evaluate whether the improved vaccine could also be effective in the treatment of TC-1 tumor of larger sizes, the treatment was delayed until day 10 instead of day 6 (**Fig. 3.6b**). Whereas both DOTAP/E7 (10 nmol) and DOTAP/ε-PA-KSS-E7 (5 nmol) vaccines given on day 6 showed an effective anti-tumor activity, mice receiving the lipopeptide formulation on day 10 exhibited a superior effect on tumor inhibition than the native E7 peptide given at the same day (* $p < 0.05$). Overall, the DOTAP/E7-lipopeptide vaccine described in this work was capable of generating efficient anti-tumor activity even at the low antigen dose compared to the original DOTAP/E7 formulation. Moreover, the improved vaccine activity was also demonstrated growth inhibition of larger tumors.

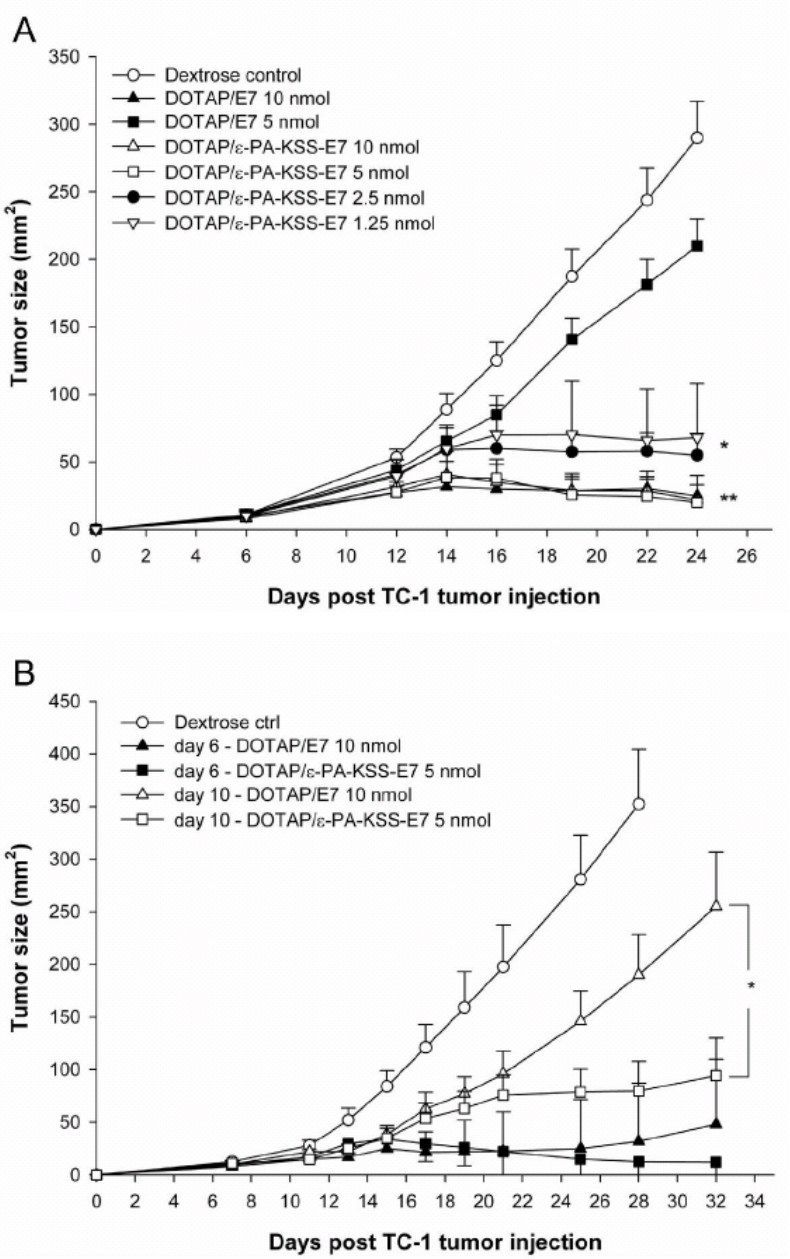


**Figure 3.6. The potency of DOTAP/E7-lipopeptide vaccine for the treatment of TC-1 tumor.**
A, TC-1 Tumor bearing mice were established as described and were given a single treatment of DOTAP/E7 or DOTAP/ε-PA-KSS-E7 formulation on day 6.Lipopeptide varying from 1.25 to 10 nmols per dose were investigated. The mean of the tumor sizes from each group at day 24 was compared to that of the group received DOTAP/E7 (5 nmol) and was analyzed by Student's t-test (* $p < 0.05$, ** $p < 0.01$, $n = 6$). B, Tumor bearing mice were treated with DOTAP/E7 (10 nmol) or DOTAP/ε-PA-KSS-E7 (5 nmol) formulation on day 6 (solid symbols) or day 10 (open symbols). The mean of the tumor sizes at day 32 was compared between the two formulations (* $p < 0.05$, $n = 6$).

## 3.4 DISCUSSION

Development of safe and effective therapeutic cancer vaccines for human use remains an urgent and unmet medical need. In this report, we demonstrate an improved liposomal lipopeptide vaccine for the treatment of HPV positive tumors. By linking to a mono-palmitic acid to KSS-elongated E7 peptide (at either α or ε position), the peptide encapsulation efficiency within liposomes was higher than that of the unmodified E7 peptide. The DOTAP/E7-lipopeptide vaccine induced an overall enhancement in generating antigen-specific CTL for eradicating HPV positive TC-1 cells (**Fig. 3.3**). When given at a reduced amount (5 nmol or less) of peptide dose to the tumor bearing mice, DOTAP/E7-lipopeptide exhibited a superior anti-tumor activity compared to the original DOTAP/E7 formulation at a full dose (10 nmol) (**Fig. 3.2** and **3.6a**). Furthermore, the therapeutic effect of DOTAP/E7-lipopeptide was also effective for suppression of tumor growth in later stages of tumor progression, suggesting applications in progressed cancer treatments (**Fig. 3.6b**).

The enhanced antigenicity and anti-tumor activity of the lipopeptide were correlated with the enhanced encapsulation of the lipopeptide in the liposomes. As shown in **Table 3.1**, the entrapment efficiency of lipopeptide reached to 90% when the peptide loading is 2.5 mol% among total lipid, whereas the native water soluble E7 peptide only incorporated into the liposome at about 25%. Similar enhanced liposome encapsulation of lipopeptides has also been reported [21-23]. In order for the peptide to be presented by the MHC class I pathway, the peptide has to enter the cytoplasm of the APC. Cationic liposomes deliver the encapsulated, but not free, peptide into the APCs, allowing the peptide to be released and later presented in the MHC class I pathway. Thus, the significantly higher the encapsulation of the peptide would

allow for increased peptide delivery and therefore, higher antigenicity, can be expected. However, there is an exception to the rule.

Our data indicates that the lipopeptide PA-E7 without the spacer sequence Lys-Ser-Ser, achieved high degree of liposome encapsulation, but did not show an enhanced anti-tumor activity compared to those with the spacer amino acids. The phenomenon was likely a result of some structural constraints where the conjugated fatty acid may cover the CTL epitope, thereby minimizing its recognition. Verheul et al. have also shown that the amino acid spacer sequences influence the immunological properties of the lipopeptide conjugates [24]. In addition to enter the endosomal pathway as exogenous soluble protein and peptides, lipopeptides must be processed to be free peptide again once entering into the cytoplasm of the APC. By adding a linker sequences, it may prevent the CTL epitope from being altered during the processing of the lipopeptide in the cytosol. Several additional spacer amino acids have been investigated thus far including CSS and CSKKK [25, 26] and they showed a stronger immunogenicity than those without a spacer sequence.

The KSS-E7 peptide formulated in DOTAP appears to be a better vaccine formulation than the native E7 peptide as demonstrated by the tumor growth inhibition and increased antigencity, although their liposome encapsulation efficiencies were comparable. Interestingly, we found that mice receiving DOTAP/KSS-E7 generated an increased functional CTL response (**Fig. 3.3 and 3.4**) as well as an enhanced anti-tumor activity (**Fig. 3.2**) compared with the native E7. It is known that the capacity of a peptide to bind and stabilize MHC class I molecules is directly correlated with its ability to induce specific CTL responses [20] [27]. Our results tempted us to study the MHC class I binding affinity of the native and the KSS-elongated E7 peptides. RMA-S cell line was originally selected from mutated cells on the basis of low cell

surface expression of MHC class I molecules and inability to present endogenous antigens. The synthesis of class I molecule heavy and light chains is normal, however the mutant cells undergoes various protein degradation routes at 37ºC, resulting in a decreased number of MHC on the surface of RMA-S cells. At 27ºC, the breakdown of MHC molecules is slow and H-2D$^b$ or K$^b$ molecules are able to appear on the cell surface at the reduced temperature [28]. The labile class I molecules at 37ºC can be stabilized by exposing cells to peptides which interact with H-2D$^b$ or K$^b$. Although the detailed mechanism is not known, our results shown in **Fig. 3.5** indicated that KSS-E7 bound with a higher affinity with H-2D$^b$, but not K$^b$ molecules than the native E7 peptide. Thus, it is possible that the enhanced antigenicity of α and ε-PA-KSS-E7 lipopeptides was due to both an enhanced liposome encapsulation and an elevated biding affinity with the H-2D$^b$ molecule for antigen presentation.

In summary, the results from this report demonstrate that the improved formulation, DOTAP/E7-lipopeptide is a potential therapeutic vaccine for the treatment of HPV positive tumors. The vaccine described here maintains the benefits of simplicity and safety from its original version, DOTAP/E7, which contains only two molecules, and the lipopeptide formulation further improves the vaccine by reducing the amount of antigen required to suppress progression of the cancer and tumor growth. Most importantly, the DOTAP/E7-lipopeptide vaccine induced an overall increased CTL activity that is essential for the enhanced tumor clearance and reduced rates of tumor recurrence. This novel formulation represents an excellent candidate for future cancer vaccine development.

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# 4.0 INDUCTION OF HLA-A2 RESTRICTED T CELL PROLIFERATION AND TUMOR-SPECIFIC CYTOTOXIC T LYMPHOCYTE RESPONSES BY A LIPOSOMAL PEPTIDE FORMULATION

Human papillomavirus (HPV) oncoproteins E6 and E7, which are constitutively expressed in the cervical carcinoma cells, are ideal targets for developing immunotherapies for treatment of existing cervical cancer. Induction of cytotoxic T lymphocytes (CTL) requires interaction with activated antigen-presenting dendritic cells (DC) which play a central role in eradicating virus infected cells and malignant tumors. In the current study, we have developed a peptide-based therapeutic cancer vaccine, DOTAP/hE7, which consists of a cationic lipid and a HLA-A2 restricted CTL peptide epitope derived from E7 protein. Our data demonstrated that the DOTAP/hE7 vaccine effectively activated human DC. *In vitro* stimulation of naïve HLA-$A2^+$ human T lymphocytes by DOTAP/hE7-activated autologous DC elicited a stronger clonal T cell proliferation and higher HPV-specific CTL responses than those stimulated with DC which had been treated with the peptide alone. Interestingly, DOTAP induced ERK1/2 phosphorylation in T cells, also resulted in T cell proliferation. In addition, T cells stimulated with DOTAP/$E7_{11-20}$ exhibited antigen specific CTL capability. *In vivo* CTL and anti-tumor activity induced by DOTAP/hE7 vaccine was also demonstrated in an HLA-A2 transgenic mouse model. Overall, the data suggested that DOTAP/hE7 has the potential to be a therapeutic cervical cancer vaccine and should be useful in clinical applications for the treatment of HPV-related neoplasia.

## 4.1 INTRODUCTION

The incidence of cervical cancer is highly associated with the infection of human papillomavirus (HPV), particularly the subtype HPV 16. Strategies to develop clinical applicable vaccines with prophylactic or therapeutic effect have been extensively studied. Although a preventative vaccine against HPV infection has already been launched in the market [1] [2], it cannot treat the existing HPV-associated malignant lesions [3]. Treatment options for cervical neoplasia are limited to cryotherapy, radiation therapy and chemotherapy [4]. Thus, development of a safe and effective therapeutic vaccine for treatment of cervical cancer remains an urgent and unmet need.

Since HPV E6 and E7 oncoproteins are consistently retained and expressed in cervical cancer cells, they are good targets for developing novel immunotherapies [5]. Many investigators have shown that induction of MHC class I restricted cytotoxic T lymphocytes (CTL) against HPV E6 or E7 play a central role in eradicating virus infected cells and malignant tumors [6]. These approaches have led to the development of several experimental therapeutic vaccines for the treatment of the cancer, including protein or peptide-based vaccines [7] [8] and DNA vaccines [9] [10]. HPV-specific CTL activity was induced in a murine model system upon receiving the vaccine. Some of these vaccines have already launched to various stages of clinical trials [11] [12].

Instead of the injectable vaccines, alternative strategies include adoptively transferring autologous E6 or E7 specific CTL to the host. Thus, patients essentially receive their own cells but not the vaccine which may cause toxicity. Studies from different groups have demonstrated that human CTL against HPV can be established by *in vitro* restimulation of peripheral blood mononuclear cells (PBMC) obtained from cervical cancer patients. Indeed, various stimulation methods using peptides [13], proteins [14], and vaccinia virus [15] have shown the ability of

inducing of primary HPV-specific CTL. No matter which method was used, HPV-specific CTL could not be detected in the PBMCs from normal donors unless DC was used as antigen-presenting cells *in vitro* [16] [17].

Previous studies from our lab have led to the development of a therapeutic cancer vaccine formulation consists of only a cationic lipid DOTAP and a peptide antigen derived from HPV 16 E7 oncoprotein [18]. Upon DOTAP stimulation, murine bone marrow derived dendritic cells (BMDC) were activated for the expression of the co-stimulatory molecules, CD80 and CD86 [19]. The activation of ERK pathway and several chemokines such as CCL2 were also observed in DOTAP-activated BMDC [20]. Animal studies suggested that DOTAP liposome alone provides both an antigen carrier and a potent adjuvant activity in inducing migration of the activated DC to the draining lymph node (DLN), thereby leading to the generation of *in vivo* antigen-specific $CD8^+$ T lymphocyte against antigen-bearing tumor cells [18].

These results prompted us to take advantages of the fact that DOTAP liposome functions as a strong DC stimulator to investigate and confirm the potency of DOTAP/E7 vaccine in human. Herein, we compared the immunogenicity of two HLA-A2 restricted CTL peptide epitopes ($E7_{11-20}$ and $E7_{86-93}$) which were encapsulated in DOTAP liposome. We demonstrate the enhanced induction of the HLA-A2 restricted HPV-specific CTL in normal human PBMC by *in vitro* stimulation using DOTAP/hE7-activated autologous DC or DOTAP/hE7 alone. The *in vivo* anti-tumor activity of DOTAP/hE7 vaccine was also confirmed using an HLA-A2 transgenic mice model.

## 4.2 MATERIALS AND METHODS

### 4.2.1 Lipids, reagents and cell lines

DOTAP was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Phorbol 12-myristate 13-acetate (PMA), ionomycin and lipopolysaccharide (LPS) were purchased from Sigma-Aldrich (St. Louis, MO). KG-1, a human myelogenous leukemia cell line, and CaSki, a human cervical carcinoma, were obtained from the Lineberger Comprehensive Cancer Center at the University of North Carolina at Chapel Hill. T2 cells which express only the HLA-A*0201 allele were used as target cells in CTL assay. TC-1/A2 cell line was generated by transducing TC-1, which is an established E7-expressing cell line, with a retrovirus encoding the HLA-A2 molecule [21]. Both of T2 and TC-1/A2 cells were kindly provided by Dr. T.C. Wu at Johns Hopkins University (Baltimore, MD). All of above tumor cell lines were maintained in RPMI-1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin (Invitrogen).

### 4.2.2 Generation of human DC

Human DC were prepared according to the protocol by Dees et al [22]. In brief, buffy coats from healthy donors were purchased from the American Red Cross (Durham, NC) and the PBMCs were separated by centrifugation on a ficoll hypaque gradient. After washing by PBS, cells were suspended in medium and allowed to adhere to the plastic dishes for 2 h. Non-adherent PBMCs were then removed from the flask. The adherent mononuclear cells were cultured with AIM-V (Invitrogen) culture media supplemented with 10% human AB serum and human recombinant

cytokines GM-CSF (800 units/ml) and IL-4 (500 units/ml) (R&D system, Minneapolis, MN). Fresh medium with cytokines were replenished to the culture every 48-72 h. On day 7, immature DC were harvested and used for phenotype analysis and antigen presentation.

### 4.2.3 Peptide Synthesis

The human $E7_{11-20}$ and $E7_{86-93}$ peptides containing an HLA-A2 restricted CTL epitope (**Table 4.1**) derived from HPV 16 E7 protein were synthesized and purified with a purity greater than 95% in Molecular Medicine Institute Peptide Synthesis Facility at the University of Pittsburgh. Ovalbumin (OVA) peptide (SIINFEKL) was a gift kindly provided by Dr. M.J. Cho at University of North Carolina at Chapel Hill.

### 4.2.4 Preparation and characterization of DOTAP/hE7

Cell culture grade water (Cambrex, Walkersville, MD) was used in all liposome preparations. Briefly, lipid films were made in a glass vial by evaporating the chloroform solution under a steady stream of dry nitrogen gas. Traces of organic solvent were removed by vacuum desiccation overnight. Lipid films were hydrated for 12 h by adding required amount of water containing E7 peptide. The suspension was sonicated in a bath type sonicator for 5 min followed by extrusion (Hamilton Co., Reno, NV) through a 100 nm membrane filter (Nuclepore, Pleasanton, CA). DOTAP/hE7 formulations were stored at 4ºC before use.

The particle size and the zeta (ζ) potential of the liposomal complexes were measured following the manufacturer's instruction using a Coulter N4 Plus particle sizer (Beckman Coulter, San Francisco, CA) and a ZetaPlus (Brookhaven Instruments, Corp., Holtsville, NY),

respectively. Peptide encapsulation was determined by the percentage of liposome-bound peptide using a previously published method [18]. The data are reported as the mean ± SD (n=3).

### 4.2.5 Antibodies and flow cytometry analysis

All anti-human antibodies used for flow cytometric analysis were purchased from BD Pharmingen (San Diego, CA). KG-1 cells or primary DC suspensions were incubated with fluorescently conjugated antibodies to surface antigens for 30 min at 4˚C. The following Abs were used: anti-CD11c (B-ly6), anti-CD80 (L307.4), anti-CD83 (HB-15e), anti-CD86 (2331) and anti-HLA-DR (L243). Isotype control Abs were used to set the background for the surface Ab labels. After washing with PBS, cells were resuspended in 300 μl stain buffer and analyzed using a BD FACSCanto digital flow cytometer (San Diego, CA).

### 4.2.6 *In vitro* induction of human primary CTL response

On day 7, immature DC were harvested and pulsed with hE7 peptide (40 μM) or DOTAP/hE7 (containing 50 μM lipid and 40 μM peptide) in the presence of β2-microglobulin (3 μg/ml) in the culture media for 4 h at 37°C. After washing by PBS twice, cells were treated with mitomycin-C at 25 μg/ml for 20 min. DC were then washed at least three times with PBS prior to mixing with lymphocytes. HLA-$A2^+$ human lymphocytes were obtained from the non-adherent PBMC after two consecutive plastic adherence procedures. On day 0, lymphocytes were cultured in a 24-well plate with autologous DC (10:1 ratio) which had been pulsed with E7 peptide or DOTAP/E7. The lymphocyte culture media contained AIM-V medium supplemented with 10% human serum and cytokine IL-7 (20 units/ml). An additional 1-mL of fresh culture medium with IL-2 (20

units/ml) was added to each well on day 2. The lymphocytes were washed and re-stimulated with E7 pulsed or DOTAP/E7-pulsed DC on day 7 and day 14 with the same preparation procedure. For induction of CTL in the absence of DC, lymphocytes were incubated with DOTAP/hE7 (containing 10 μM hE7) in the culture medium. Three days after the third stimulation, images of the lymphocyte cultures were taken using a Nikon phase contrast microscope. The IFN-γ level in the culture supernatant was measured by BD ELISA Set (BD Pharmingen) according to manufacturer's instruction.

Seven days after the last restimulation, T lymphocytes were harvested and used as effectors in CTL assay. In brief, T2 or CaSki target cells were labeled with PKH-67 (Sigma-Aldrich) according to manufacturer's instruction. The mixture of effectors and labeled targets were plated into a 96-well U-bottom plate at various effector:target (E:T) ratios and the cell lysis reactions were carried out for 4 h at 37°C. The cells were then harvested and stained with propidium iodide (Sigma Aldrich) prior to flow cytometric analysis. The percentage of E7-specific lysis was determined by the proportion of PI positive cells within the FL1 (PKH-67) positive region.

### 4.2.7 Western blot analysis

Human lymphocytes ($2 \times 10^6$ cells/ml) were treated with DOTAP at 50 μM in the complete medium and the cell lysate was collected at different time points after treatment. Equal amount of total cellular protein (50 μg) was resolved on polyacrylamide/SDS gels (Bio-Rad, Hercules, CA) and then transferred to a polyvinylidene difluoride membrane. The membrane was blocked with 5% non-fat milk in PBS for 1 h and incubated overnight with primary Ab at 4°C. After

washing with PBS three times, the membrane was incubated with horseradish peroxidase (HRP) conjugated secondary Ab for 1 h. The enzyme activity associated with the protein bands was detected using ECL Plus™ (Amersham, Pittsburgh, PA) followed by autoradiography.

### 4.2.8 Mice and immunizations

All work performed on animals was in accordance with and approved by the institutional IACUC. Five to six week old HLA-A*0201 transgenic mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Subcutaneous tumors were established by injecting TC-1/A2 cells ($10^6$ cells) into the hair-trimmed flank of the mouse on day 0. Mice were treated with a DOTAP/hE7 formulation subcutaneously (s.c.) at the other side of flank on day 6. The size of the tumor was measured using a caliper two or three times a week. Tumor size was determined by multiplying the two largest dimensions of the tumor.

### 4.2.9 Analysis of *in vivo* CTL response

Mice were immunized twice with DOTAP/hE7 with one week interval between each dose. *In vivo* CTL response was assessed 7 days after the last immunization. Spleen cells from syngenic mice were RBC lysed followed by pulsing with 10 μM $E7_{11-20}$, $E7_{86-93}$ or without peptide in complete medium for 1 h at 37°C. Three spleen cell populations were stained with equal amounts of 2 μM PKH-26 (Sigma-Aldrich) according to manufacturer's instruction. The $E7_{11-20}$-pulsed, $E7_{86-93}$-pulsed and the unpulsed populations were then loaded with 5, 0.5 and 0.05 μM CFSE (Molecular Probe), respectively, at 37°C for 15 min. The three populations were mixed together (1:1:1) for tail vein injection to the control or the immunized mice ($10^7$ cells per mouse).

At 16 h after injection, spleen cells from the recipient mice were isolated and single cell suspension were prepared prior to flow cytometric analysis. The numbers of $CFSE^{high}$, $CFSE^{medium}$ and $CFSE^{low}$ population were determined and the *in vivo* E7 specific lysis percentage was enumerated according to a published equation [23].

### 4.2.10 Statistical analysis

Data were analyzed statistically using a two-tailed Student t-test. Difference in data was considered statistically significant when p value was less than 0.05.

## 4.3 RESULTS

### 4.3.1 Characterization of DOTAP/hE7 complexes

DOTAP/hE7 formulations used in this study consisted of a cationic lipid DOTAP and a peptide antigen containing an HLA-A2 restricted CTL epitope derived from the E7 protein of HPV 16. In **Table 4.1**, the particle size and zeta potential of the DOTAP/hE7 complexes were similar to those of DOTAP liposome alone. The peptide entrapment efficiency in the liposome was also compared between two HLA-A2 restricted peptides. $E7_{11-20}$ showed a significantly higher incorporation in the liposome than $E7_{86-93}$, likely owing to the stronger association between the more negatively charged peptide ($E7_{11-20}$) and the cationic liposome as compared to the less charged peptide ($E7_{86-93}$).

**Table 4.1. Physicochemical properties and peptide entrapment efficiency of DOTAP/hE7 complexes**

| | Peptide seq. | Particle size (nm) | Zeta potential (mV) | Entrapment efficiency (%) |
|---|---|---|---|---|
| DOTAP liposome | - | 109.8 ± 33.4 | 51.2 ± 4.0 | - |
| DOTAP/$E7_{11-20}$ | YMLDLQPETT | 125.3 ± 40.1 | 50.1 ± 3.5 | 63.6 ± 5.9 |
| DOTAP/$E7_{86-93}$ | TLGIVCPI | 124.8 ± 32.5 | 49.3 ± 4.2 | 17.1 ± 5.4 |

n = 3 per group

### 4.3.2 Activation of human DC by DOTAP/hE7 formulation

Since the activated DC initiate the DC-T cell interactions by presenting MHC bound peptide antigen to T cells [24], it is important to assess whether a peptide vaccine could interact with DC and lead DC to activation. We first tested our DOTAP/hE7 formulation for activation of KG-1, which is a human myelogenous leukemia cell line. KG-1 cells have been established to possess the capacity to mature into both macrophages and dendritic-like cells [25] and they have been used to examine the processes involved in dendritic cell precursor maturation [26]. As shown in **Fig. 4.1a**, upon treatment with PMA and ionomycin, conditions which induce differentiation of $CD34^+$ hematopoetic progenitor cells into dendritic cells [27], KG-1 cells expressed the DC lineage markers, CD11c and CD83, as well as the co-stimulatory molecules such as CD80 and CD86, compared to the untreated control. In addition, KG-1 cells stimulated with DOTAP/$E7_{11-20}$ exhibited a similar extent of differentiation as those induced by phorbol ester.

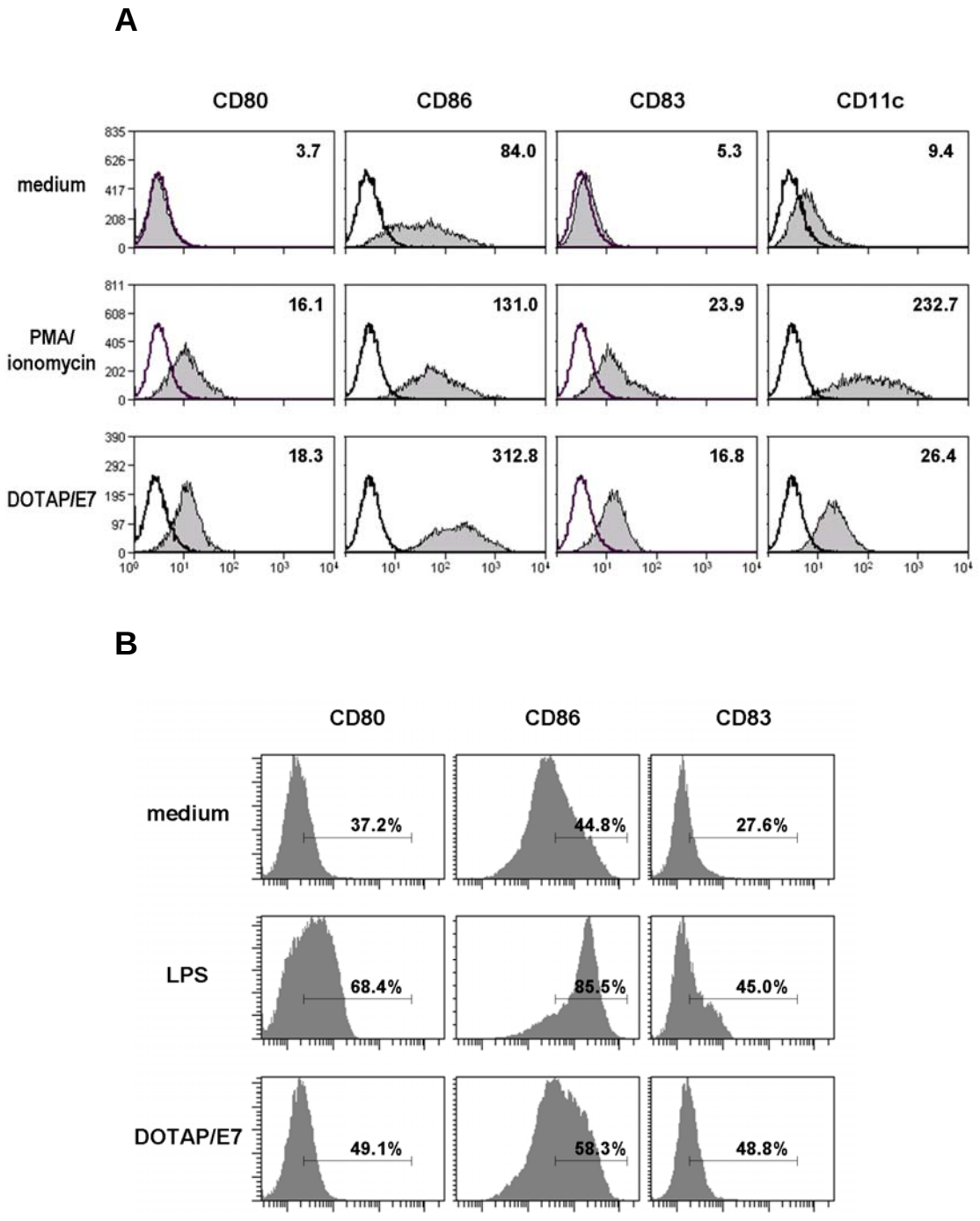


**Figure 4.1. Activation of human dendritic cells by DOTAP/hE7.**
A, KG-1, human DC-like cells were cultured in medium alone, PMA (10 ng/ml) plus ionomycin (100 ng/ml) for 4 days or DOTAP/$E7_{11\text{-}20}$ (containing lipid 50 μM) for 1 day. Cells were harvested and stained for surface antigens prior to the flow cytometric analysis. Ag-specific staining is shown in grey area and the isotype matched control is indicated by the open area. The numbers shown on the histograms represent the mean fluorescence intensity of the indicated antigen. B, Primary human immature DC were prepared as described. On day 7, DC were treated with medium alone, LPS (200 ng/mL), DOTAP/$E7_{11\text{-}20}$ (containing lipid 50 μM) for 16 h and analyzed by flow cytometry. The numbers indicate the relative percentage of the positive populations.

We further investigated the effect of DOTAP on the activation of human primary DC (**Fig. 4.1b**). After *in vitro* culture with GM-CSF and IL-4 for 7 days, human immature DC with $CD11c^+$ phenotype were treated with LPS or DOTAP/E7 for 16 h prior to FACS analysis for the activation markers on the cell surface. Human primary DC could be activated by both LPS and the DOTAP/E7 formulation, which led to the further maturation of DC toward the up-regulation of surface markers such as CD80, CD86 and CD83. Similar results were obtained in three different batches of human DC preparation.

### 4.3.3 Induction of human T cell proliferation by DOTAP/hE7-pulsed DC

Successful eradication of cancerous or infected cells *in vivo* by induction of cell-based immunity requires not only antigen delivery and activation of dendritic cells, but concomitant presentation of antigen to T-cells, and subsequent clonal expansion of antigen-specific cytotoxic T cell responses. To assess whether DOTAP/hE7 is capable of promoting antigen specific T-cell expansion, isolated HLA-$A2^+$ lymphocytes (after depletion of plastic-adhered monocytes) were treated with autologous DC which had been pulsed with E7 peptide alone or DOTAP/hE7 formulation followed by mitomycin-C treatment. The stimulation was repeated at least three times with a 7-day interval. As shown in **Fig. 4.2b**, lymphocytes treated with unpulsed DC exhibited a minimal proliferation in the culture compared to the medium control (**Fig. 4.2a**) while lymphocytes treated with peptide-pulsed DC (**Fig. 4.2c** and **4.2e**) displayed few or moderate colonies of expanding T-cells. Interestingly, a strong T cell proliferation was observed after stimulation with DC which had been activated by DOTAP/$E7_{11\text{-}20}$ or DOTAP/$E7_{86\text{-}93}$ (**Fig. 4.2d** and **4.2f**).

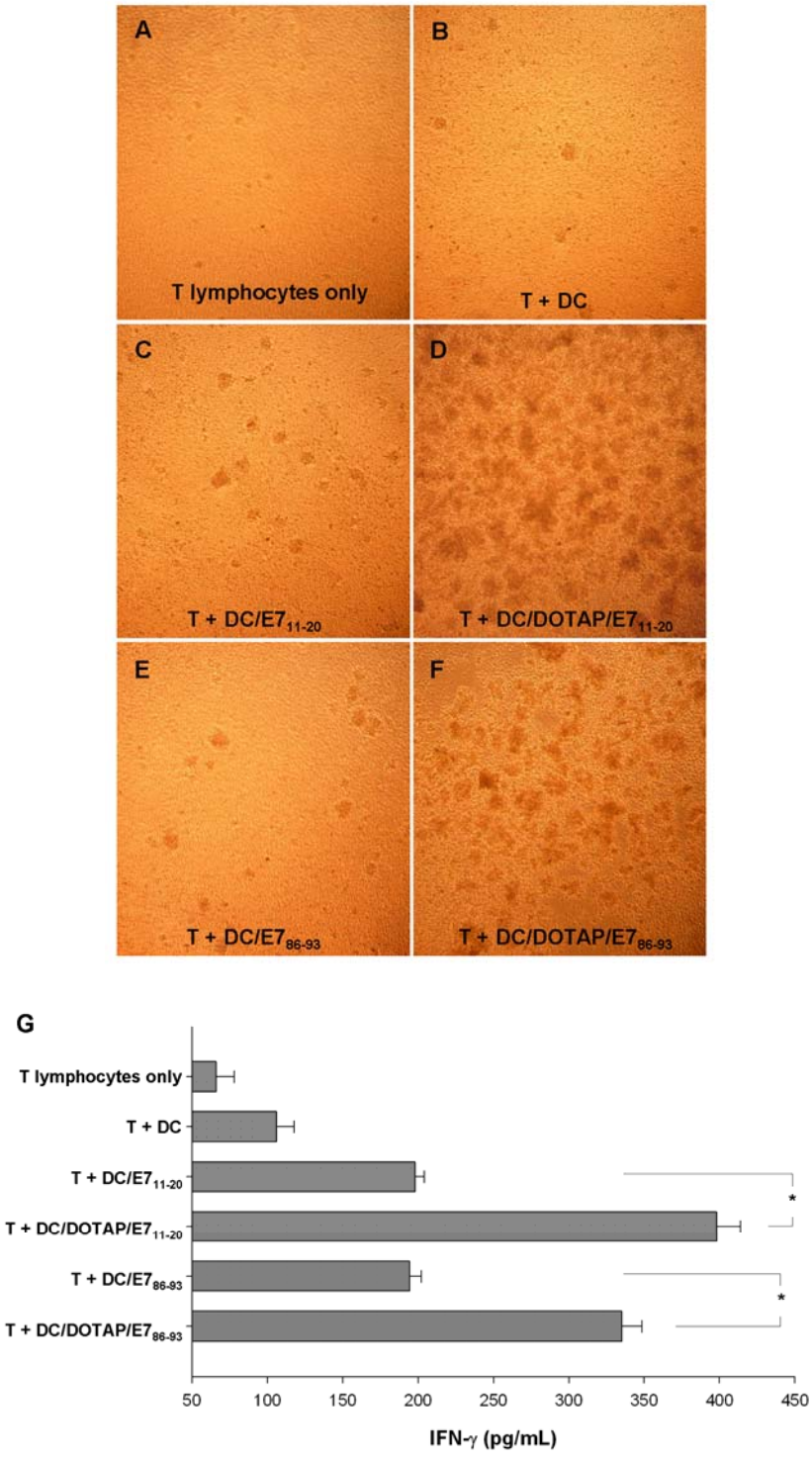


**Figure 4.2. Induction of Ag-specific T cell proliferation by incubating lymphocytes w/ DOTAP/E7-pulsed DC.** Human T lymphocytes obtained from the HLA-A2 healthy donor were incubated with A, medium only; B, unpulsed autologous DC; C, $E7_{11-20}$ pulsed DC; D, DOTAP/$E7_{11-20}$ pulsed DC; E, $E7_{86-93}$ pulsed DC and F, DOTAP/$E7_{86-93}$ pulsed DC. Three days after the third stimulation, the images of the culture were taken by a phase contrast microscope. G, IFN-γ secretion in the culture was measured by ELISA ($n = 3$, $*P < 0.05$)

Further confirmation of the antigen specificity for the expanding T cells was provided by IFN-γ secretion in the culture (**Fig. 4.2g**). Lymphocytes incubated with DOTAP/$E7_{11-20}$ or DOTAP/$E7_{86-93}$ pulsed DC showed a significant increase in IFN-γ production compared to those incubated with unpulsed or peptide-pulsed DC. The results indicated that the interaction between DOTAP/E7-activated DC and autologous lymphocytes underwent extensive expansion of T-cell colonies in the culture.

### 4.3.4 *In vitro* induction of tumor-specific human primary CTL by DOTAP/E7-activated autologous DC

The clonally expanded T lymphocytes induced with DOTAP/E7-activated DC were tested for antigen-specific cytotoxicity using a flow cytometry based method. Lymphocytes were stimulated with DC which had been pulsed with $E7_{11-20}$, DOTAP/$E7_{11-20}$ (**Fig. 4.3a**) or with $E7_{86-93}$, DOTAP/$E7_{86-93}$ (**Fig. 4.3b**). Seven days after the last stimulation, CTL assay was performed by mixing T cell populations with the fluorescently labeled target cells. Both of the $E7_{11-20}$-CTL and $E7_{86-93}$-CTL showed effective killing of HLA-$A2^+$, HPV $16^+$ CaSki cells (closed symbols) but not HLA-$A2^+$, HPV $16^-$ T2 cells (open symbols). More importantly, the human T cells stimulated with the DOTAP/h$E7_{11-20}$-activated DC exhibited a stronger antigen-specific CTL effect than those incubated with $E7_{11-20}$ peptide pulsed DC ($P < 0.01$), thereby confirming that the clonal expanded T cells by the DOTAP/E7-activated DC were functional, antigen-specific CTL.

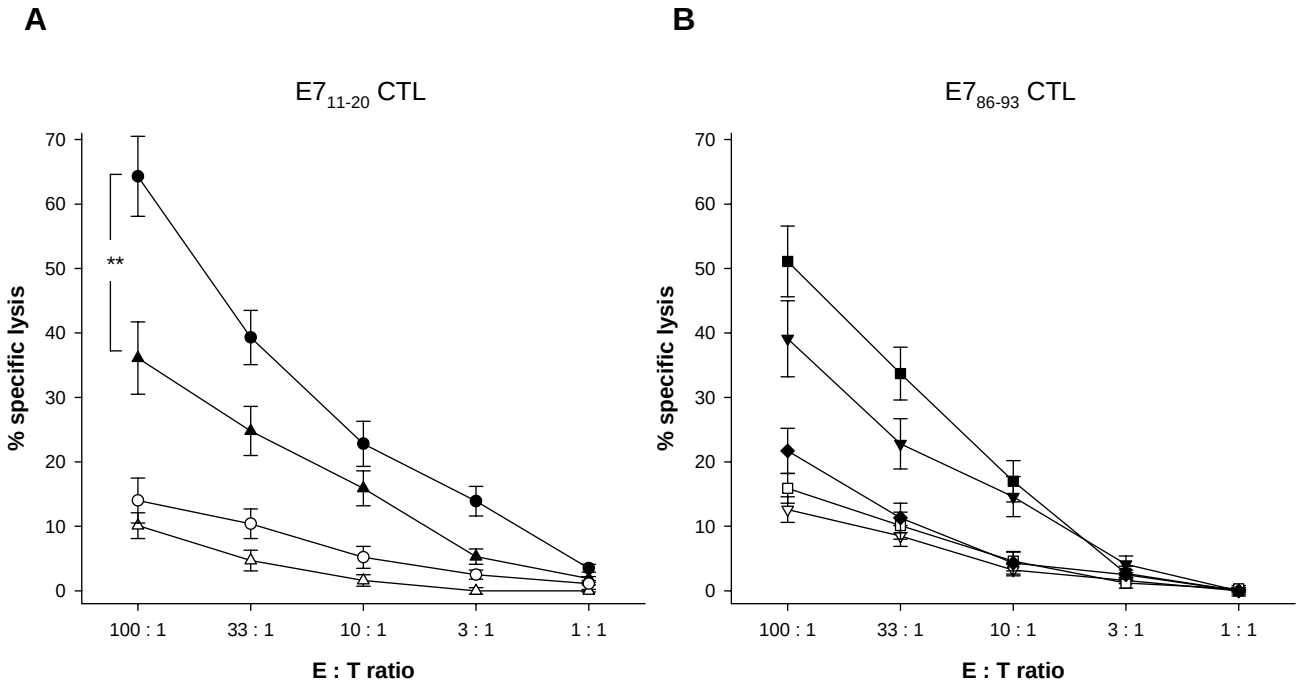


**Figure 4.3. *In vitro* induction of HPV-specific cytotoxicity of human $E7_{11-20}$ (A) and $E7_{86-93}$ (B) CTLs.** Cytotoxicity was tested 1 week after the third stimulation of HLA-$A2^+$ T lymphocytes with $E7_{11-20}$ pulsed DC (▲); DOTAP/$E7_{11-20}$ pulsed DC (●); $E7_{86-93}$ pulsed DC (▼); DOTAP/$E7_{86-93}$ pulsed DC (■) and unpulsed DC (♦). CTLs were measured using a flow cytometry based method against CaSki (closed symbol) and T2 (open symbol) target cells. The data were shown as mean ± SD (n = 3).

### 4.3.5 DOTAP/E7 mediated proliferation and ERK activation in T cells

As some of the recent developed adjuvants can directly act on T cells [28], we found that DOTAP/hE7 also interacts with human T lymphocytes, leading to clonal expansion and T cell activation. Enriched human lymphocytes obtained from an HLA-$A2^+$ healthy donor were stimulated by DOTAP or DOTAP/hE7 which were directly added into the culture. The stimulation was repeated three times with a 7-day interval. Three days after the third stimulation, lymphocytes treated with DOTAP (**Fig. 4.4b**) or DOTA/$E7_{11-20}$ (**Fig. 4.4d**) showed extensive expansion of T cell colonies in culture in contrast to no clonal expansion in medium control (**Fig. 4.4a**) and few colonies of expanding T cells in treating with $E7_{11-20}$ peptide alone (**Fig. 4.4c**). The

DOTAP-mediated T cell activation was further confirmed by ERK phosphorylation in T cells. As shown in **Fig. 4.4e**, human T cells treated with DOTAP at 50 μM induced a strong ERK phosphorylation with a maximum signal at 10 min after the treatment. The results suggested that DOTAP has a direct impact on T cell activation via a MAP kinase mediated cell proliferation.

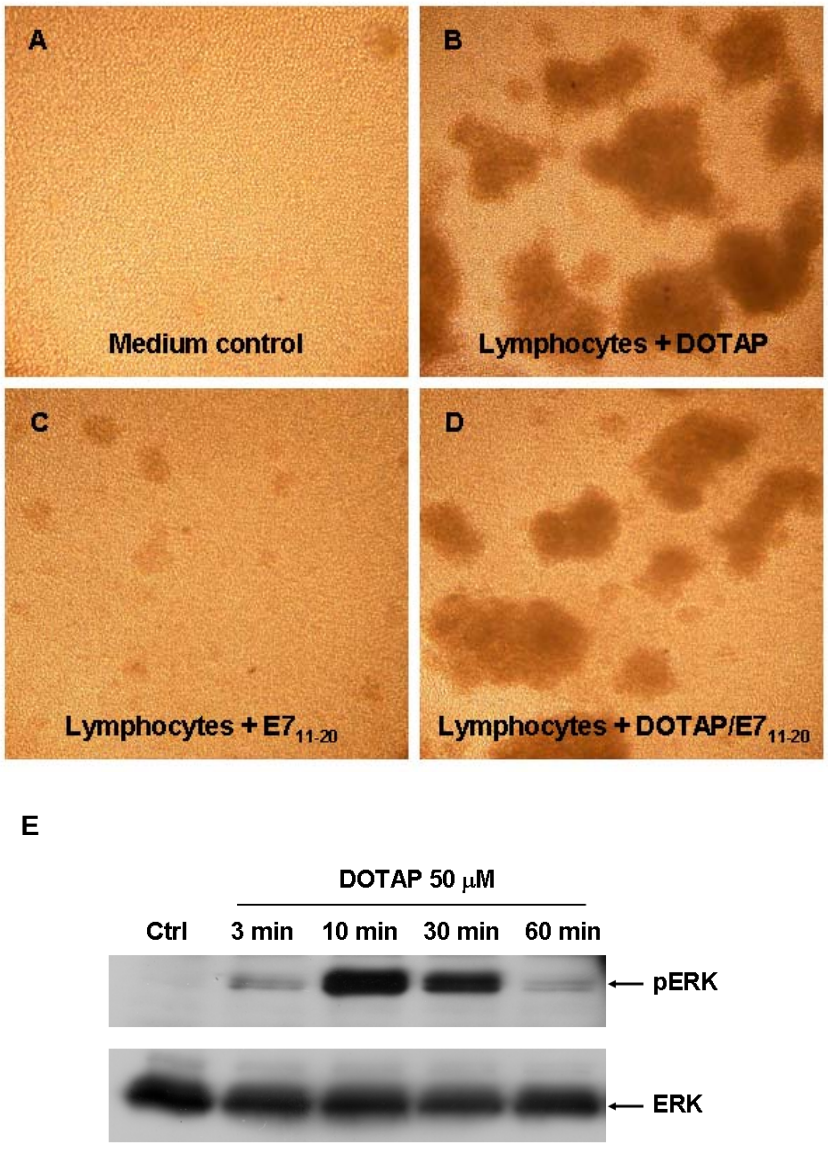


**Figure 4.4. DOTAP-induced human lymphocyte proliferation and activation.**
Human lymphocytes obtained from the HLA-A2 healthy donor were incubated with A, medium only; B, DOTAP (25 μM) alone; C, $E7_{11-20}$ (10 μM); D, DOTAP/$E7_{11-20}$ (containing 25 μM lipid and 10 μM peptide). Three days after the third stimulation, the images of the culture were taken by a phase contrast microscope. E, DOTAP-induced ERK phosphorylation was detected by Western blot analysis.

### 4.3.6 Antigen specificity of CTL response induced by DOTAP/E7$_{11-20}$

Human T lymphocytes showed clonal expansion in response to both DOTAP and DOTAP/E7$_{11-20}$ treatments (**Fig. 4.4**). These cells were tested for CTL response in killing of E7$_{11-20}$ pulsed T2 (HLA-A2$^{+}$, E7$^{+}$) and OVA peptide pulsed T2 (HLA-A2$^{+}$, E7$^{-}$) target cells. As shown in **Fig. 4.5a**, DOTAP/E7$_{11-20}$ (■) stimulated T cells at high E:T ratio were capable of killing T2 targets which had been pulsed with E7$_{11-20}$ but not T2 pulsed with an irrelevant peptide (**Fig. 4.5b**). The specific killing capability was not observed when T cells were treated with medium, peptide alone nor DOTAP alone. The confirmation of antigen specificity of DOTAP/E7$_{11-20}$ induced CTL response was also provided by their ability to lyse CaSki cervical cancer cell line (data not shown). The experiment was repeated three times with different donor blood cells and the results were similar to each other.

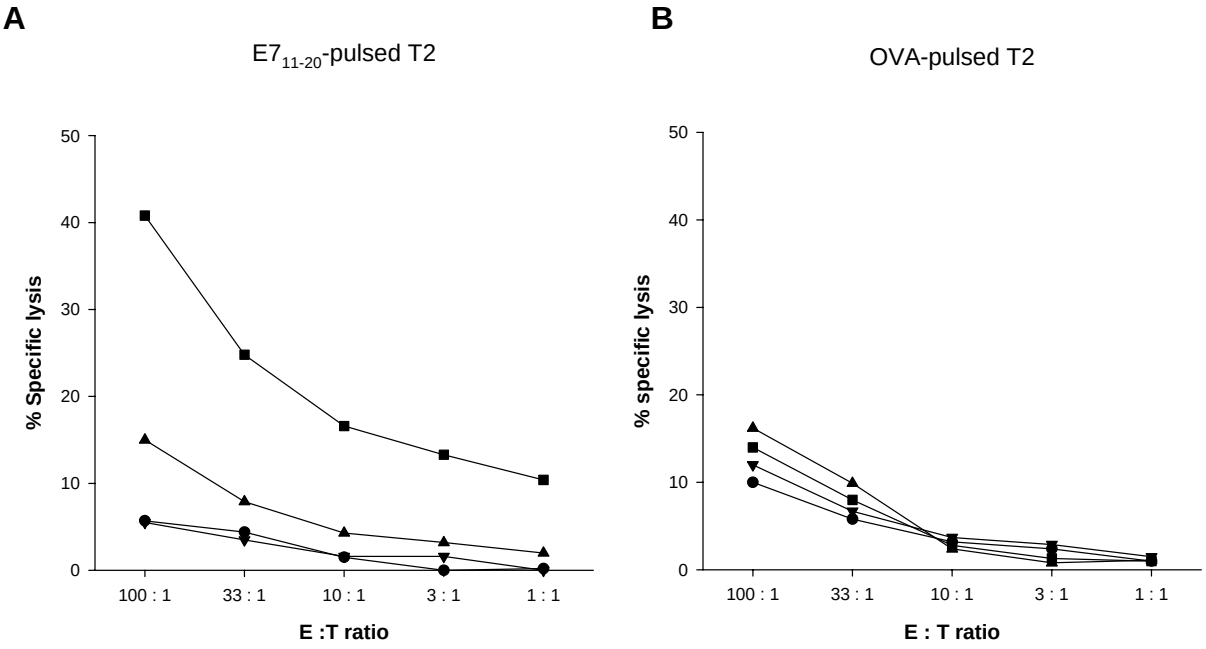


**Figure 4.5. Antigen specificity of the CTL response induced by DOTAP/hE7.**
CTL response was measured using a flow cytometry based method against E7$_{11-20}$ pulsed T2 (A) and OVA peptide pulsed T2 (B). Cytotoxicity was carried out at 1 week after the third stimulation of HLA-A2$^{+}$ T lymphocytes with medium only (●); DOTAP alone (▼); E7$_{11-20}$ alone (▲); or DOTAP/E7$_{11-20}$ (■). The data were shown as mean ± SD (n = 3). One representative data from three performed was shown.

### 4.3.7 Augment of *in vivo* CTL responses in HLA-A2 transgenic mice after vaccination with DOTAP/hE7 formulations

To access the *in vivo* immunogenicity of DOTAP/hE7, HLA-A*0201 transgenic mice were vaccinated with the DOTAP/$E7_{11-20}$ or DOTAP/$E7_{86-93}$ formulation and assayed for the *in vivo* CTL activity. A mixture of target cells containing $E7_{11-20}$ pulsed ($CFSE^{high}$), $E7_{86-93}$ pulsed ($CFSE^{medium}$) and unpulsed ($CFSE^{low}$) syngenic spleen cells was tail vein injected into the untreated, DOTAP/$E7_{11-20}$ vaccinated or DOTAP/$E7_{86-93}$ vaccinated A2 transgenic mice and allowed *in vivo* killing for 16 h prior to the FACS analysis. As shown in **Fig. 4.6**, the percentage of $E7_{11-20}$ specific killing in mice that were immunized with DOTAP/$E7_{11-20}$ formulation was more than 80%, while in the groups received DOTAP/$E7_{86-93}$ formulation showed about 35% of $E7_{86-93}$ specific killing. These results showed that DOTAP/hE7 formulation induced an HLA-A2 restricted, antigen-specific CTL *in vivo* and the DOTAP/$E7_{11-20}$ formulation elicited a higher CTL activity than the DOTAP/$E7_{86-93}$ formulation.

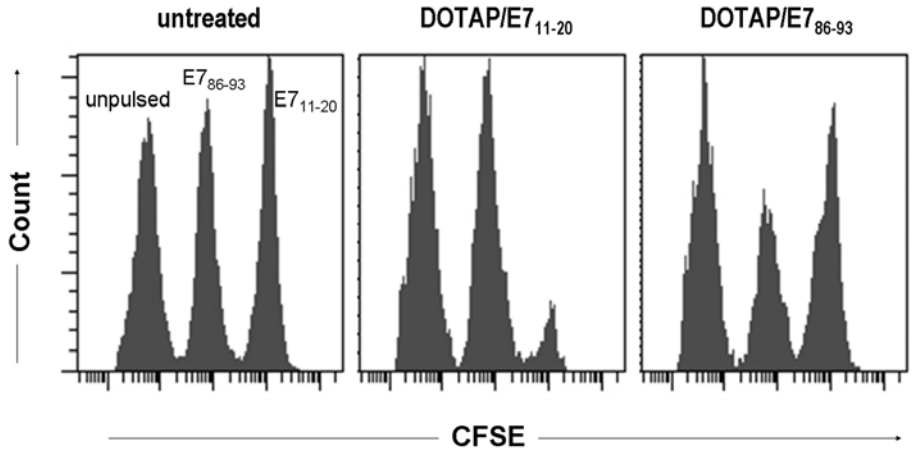


**Figure 4.6. Peptide-specific CTL were induced *in vivo* in HLA-A2 Tg mice with DOTAP/hE7 immunization.** Seven days after the second immunization, the representative mice from the untreated, DOTAP/$E7_{11-20}$ or DOTAP/$E7_{86-93}$ treated groups were iv injected with an equivalent amount of $E7_{11-20}$ pulsed ($CFSE^{high}$), $E7_{86-93}$ pulsed ($CFSE^{medium}$) and unpulsed ($CFSE^{low}$) spleen cells from a syngenic donor. The *in vivo* CTL response was carried out for 16 h and the spleen cells from the recipient mice were analyzed by flow cytometry.

### 4.3.8 Therapeutic effect of DOTAP/E7$_{11-20}$ against TC-1/A2 established tumor

The therapeutic effect of DOTAP/E7$_{11-20}$ against the established TC-1/A2 tumor was confirmed in HLA-A*0201 transgenic mice (**Fig. 4.7**). TC-1/A2 was generated by transducing TC-1, an established E7-expressioing tumor cell line with a retrovirus encoding the HLA-A2 molecule [21]. TC-1/A2 tumor was established by subcutaneouly injecting $10^6$ tumor cells into the A2 transgenic mice on day 0. As shown in **Fig. 4.7**, mice treated with a single dose of DOTAP/E7$_{11-20}$ on day 6 exhibited a significant reduction in tumor burden compared with the untreated mice (P = 0.002 on day 29). These data indicated that the DOTAP/E7$_{11-20}$ therapeutic vaccine was capable of inducing antigen-specific CTL activity responsible for an anti-tumor response in the presence of a growing tumor.

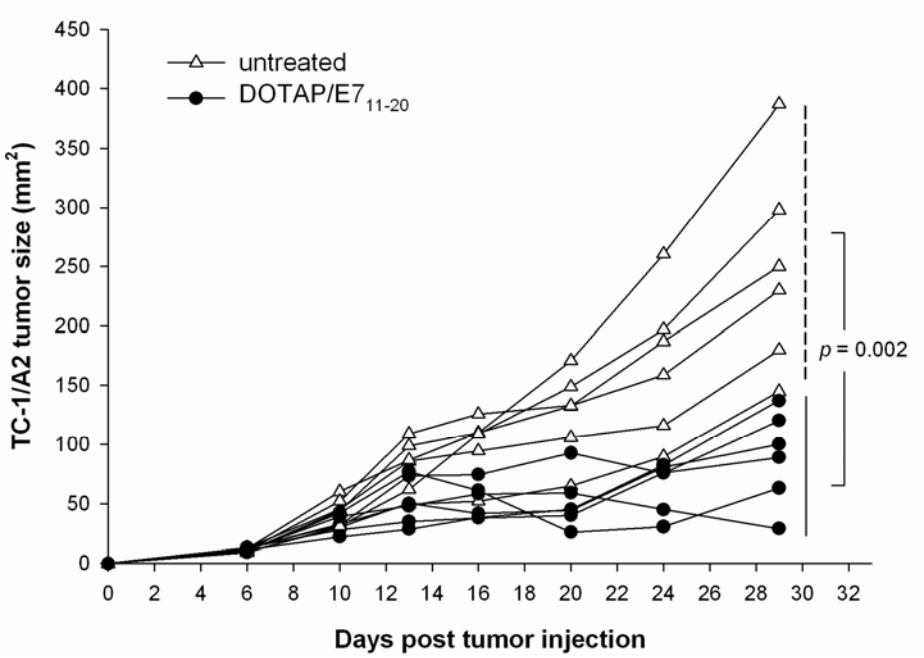


**Figure 4.7. Kinetics of TC-1/A2 tumor growth in HLA-A2 Tg mice with a single treatment of DOTAP/E7$_{11-20}$.** A2 transgenic mice (n = 6) were subcutaneously injected with $10^6$ TC-1/A2 tumor cells on day 0 and the mice were left untreated or treated with DOTAP/E7$_{11-20}$ on day 6. Tumor sizes were measured two or three times per week. Each line represents the tumor growth of an individual animal. Tumor size of the treated group on day 29 was compared to that of the untreated control and was analyzed statistically.

## 4.4 DISCUSSION

Previous reports in our lab have shown the potency and the possible vaccine mechanism of DOTAP/E7 in a mouse model system, in which the murine H-2D$^b$ restricted E7 peptide (a.a. 49-57) epitope was studied [18, 20]. The data presented in this report summarizes studies performed to extend our understanding of the DOTAP/E7 vaccine to human *in vitro* and *in vivo* systems by formulating HLA-A2 restricted CTL epitope ($E7_{11-20}$ or $E7_{86-93}$) into the DOTAP liposomes. *In vitro* testing in activation of human dendritic-like KG-1 cells and primary human DC (**Fig. 4.1**) indicated that DOTAP cationic lipids are potent immunostimulators, promoting dendritic cell activation toward expression of co-stimulatory molecules which were required for T-cell recognition and antigen presentation. We have also demonstrated that human DC, upon activation by DOTAP/hE7 liposomes, promoted significant proliferation of human T-cells *in vitro* (**Fig. 4.2**). In addition, human T-cells stimulated by DOTAP/hE7-activated autologous DC were capable of mounting a tumor-specific cytotoxic response *in vitro* (**Fig. 4.3**). Our data also showed the capability of DOTAP/hE7 to interact with human T cells, leading to clonal expansion and antigen-specific CTL response (**Fig. 4.4** and **Fig. 4.5**). For *in vivo* demonstration, HLA-A*0201 transgenic mice immunized by DOTAP/hE7 induced an *in vivo* antigen-specific cytotoxic T-cell response (**Fig. 4.6**). Finally, upon injecting a single dose of DOTAP/$E7_{11-20}$ to the TC-1/A2 (HLA-A2$^+$, HPV E7$^+$) tumor-bearing transgenic mice, suppression of tumor growth resulted as compared to the untreated animals (**Fig. 4.7**).

The generation of mature human DC involves at least two stages [29]. The first stage includes the differentiation of CD34$^+$ hemopoietic progenitors to the immature CD11c$^+$/CD83$^+$ DC lineage cells with the stimulation of fresh cytokines (e.g. GM-CSF and IL-4) replenished every 48-72 h [30] [31]. The immature DC is highly capable for antigen uptake but relatively

poor in activating T cells. The second stage of the process involves the activation of the immature, resting DC to the mature, fully stimulatory antigen-presenting cells (APC) by an addition of a danger signal, such as TNF-α and LPS. Along this process, DC loses antigen-capturing ability but is much more potent in activating T cells [32]. The data shown in **Fig. 4.1** indicated that DOTAP was sufficient to trigger DC activation compared to the potent stimuli of phobol ester PMA, which activates protein kinase C, plus a calcium ionophore ionomycin [33]. Upon DOTAP stimulation, the danger signal which is required for DC maturation was likely provided by the production of the reactive oxygen species (ROS) in DC [18]. In addition, the subsequent signals mediated by DOTAP stimulation, such as CCL2 chemokines and ERK activation also contributed to promoting survival of DC [20] [34].

Naïve $CD8^+$ T cells require interaction with antigen-presenting cells to achieve full activation of cell-mediated immune responses [35]. Presentation by mature DC results in antigen-specific CTL, however, antigen presented by inactivated DC to T cells induces tolerance [36] [37]. To avoid tolerance, during the process of *in vitro* DC-naïve T cell incubation, it is critical to have antigen-presenting DC fully activated prior to encounter with T cells. Our data shown in **Fig. 4.2** and **Fig. 4.3** also supported the notion that DC being activated by DOTAP/hE7 elicited a stronger clonal T cell proliferation and higher HPV-specific CTL responses as compared to those pulsed only with E7 peptide.

It has been extensively studied that several adjuvants of bacterial origins are designed to comprise pathogen-associated molecular patterns (PAMP) which are recognized by toll like receptors (TLR) on DC or macrophages [38]. The well-tolerant and effective adjuvants, monophosphoryl lipid A (MPL) and its chemical mimetic RC529 have been shown to signal through TLR4 [39] [40]. MPL activates human monocyte-derived DC at high dose of 100 μg/ml.

Similar to its parental molecule LPS, MPL also acts on T cells directly [28]. Our data showed that DOTAP cationic lipid activated human DC at 50 μM (~35 μg/ml) and it also induced ERK1/2 phosphorylation in T cells, leading to T cell proliferation (**Fig. 4.4**). Unlike MPL and LPS which induce a strong NF-κB production through TLR signaling, DOTAP does not induce NF-κB activity nor TNF-α production [41] [19]. Our result is opposite to the traditional adjuvant concept that the more inflammatory the adjuvant is, the better immunostimulatory responses.

Unlike some soluble adjuvants, DOTAP delivers the antigen (e.g. E7) into APC (e.g. DC) via a particulate manner, while it stimulates DC activation/maturation at the same time. The mechanism of the interaction between DOTAP/E7 and DC is fairly similar to that of multivesicles or exosomes which are produced by APC [42] [43]. Indeed, exsomes are membrane-bound molecules with the size less or equal to 100 nm. Similar to our previous results demonstrating that DOTAP/E7 complex is mainly taken up by DC *in vivo* [18]; exosomes also are internalized and processed by immature DC [44]. Recent studies show that exosomes exchange peptide-MHC complexes between DC, and the eradication of established tumor is observed following a treatment of DC-derived exosomes [45] [46].

Results presented in **Fig. 4.4** showed that both DOTAP and DOTAP/$E7_{11-20}$ induced extensive colonies of expanding T cells. However, only T cells stimulated with DOTAP/$E7_{11-20}$ exhibited the antigen specific CTL capability (**Fig. 4.5**). This unusual observation suggests that DOTAP not only interacts directly with T cells, it delivers the antigen to T cells while activates them at the same time. The detail mechanism how DOTAP introduces antigen to T cells is unknown. Since the activated T cells express co-stimulatory molecules (CD80 and CD86) on the surface, the antigen presentation may occur through the interaction between two T cells. We also

cannot rule out that the DOTAP-activated B cells existing in the culture may also play a role in the antigen presentation to T cell.

Both of the HLA-A2 restricted peptides, $E7_{11-20}$ and $E7_{86-93}$ which were predicted by Kast et al. [47] elicited immunogenicity when formulated in our liposomal vaccine formulation. The activity of the *in vitro* induced $E7_{11-20}$ CTL, however, appeared to be superior to that of $E7_{86-93}$ CTL in killing CaSki cervical cancer cells (**Fig. 4.3**). A supportive result can be seen in the HLA-A2 transgenic mouse model (**Fig. 4.6**) that *in vivo* CTL response generated by DOTAP/$E7_{11-20}$ formulation was stronger than that induced by DOTAP/$E7_{86-93}$ in eliminating peptide-pulsed targets. Some of the previous reports showed that A2 transgenic mice vaccinated with E7 protein or E7-producing DNA vaccine did not generate $E7_{86-93}$ specific CTL responses [21] [48]. Although the reduced immunogenicity of $E7_{86-93}$ observed in this report may be due to the lower peptide entrapment efficiency in the liposome (**Table 4.1**), there is also a possibility that human cervical tumor cells may not always naturally present this epitope [14] [49].

The clinical application of DOTAP/E7 vaccine in human is substantially broader than our previous vaccine version, LPD/E7, which consists not only lipid and peptide antigen but also CpG-containing plasmid DNA and polycations for DNA condensation [50]. Despite the possible side effects caused by the bacterial CpG motifs, it is also costly to manufacture clinical grade DNA. The *in vivo* cell-mediated immune responses elicited by subcutaneously injection of DOTAP/hE7 to the HLA-A2 transgenic mice (**Fig. 4.6** and **Fig. 4.7**) indicated the *in vivo* efficacy and potentials of DOTAP/hE7 vaccine being administered directly to the patients.

We also observed that the anti-tumor effect of DOTAP/$E7_{11-20}$ to the TC-1/A2 tumor (**Fig. 4.7**) was not as strong as that of DOTAP/$E7_{49-57}$ to the murine TC-1 tumor model [18], likely owing to the insufficient expression of HLA-A2 molecules on the surface of TC-1/A2 cell (data

not shown). The observation was in accordance to other previous studies using TC-1/A2 or HLF16, a similar HLA-$A2^+$ HPV $E7^+$ tumor cell line [51] [52].

In summary, the data in this report demonstrated that the DOTAP/hE7 vaccine induced cell-mediated anti-tumor immune response as seen in murine model studies can be applied in human *in vitro* and in a transgenic murine model. Further testing of DOTAP/hE7 vaccine on PBMC obtained from cervical cancer patients would greatly improve the understanding of the dosing and the efficacy of the vaccine prior to the clinical application.

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# 5.0 DISCUSSION

## 5.1 CONCLUSION AND FUTURE DIRECTIONS

The evidences provided in this report demonstrate a great potential of DOTAP/E7 therapeutic vaccine for future clinical trials. The value of this work lies in the simplification of the vaccine design and the clarification of the immune responses induced by this simple vaccine. Our unpublished data show that mice were 100% protected by DOTAP/E7 immunization from the TC-1 tumor challenge, indicating DOTAP/E7 vaccine is also effective as a preventative vaccine against $HPV^+$ nascent tumor.

The data presented in **Chapter 4** suggest at least three feasible clinical applications of the DOTAP/hE7 vaccine. First approach is to inject vaccines directly into the patients. We have demonstrated in an HLA-A2 transgenic mouse model that immunization with DOTAP/hE7 generates antigen-specific CTL response which translates to the anti-tumor activity in the tumor ($HLA\text{-}A2^+$, $HPV^+$) bearing mice. Secondly, an improved DC vaccine can be achieved by *ex vivo* stimulating autologous DC with DOTAP/hE7 formulation. In addition to the delivery of the antigen to DC, our data show that DOTAP also activates human DC toward the expression of CD80, CD86 and CD83 markers, which agrees with the previous observation obtained from a mouse model. Furthermore, the naïve human T cells can be stimulated by DOTAP/hE7 activated DC, resulting in clonal expansion and exhibited antigen specific CTL activity. Since maturation

of antigen-primed DC results in an enhanced antigen presentation capability as well as immunogenicity compared to the immature DC [1], several recent strategies in developing cancer immunotherapy involve the use of mature DC [2]. The most characterized DC-maturing agents are inflammatory cytokines such as corss-linking of TNF superfamily [3]. Unlike these inflammatory agents, DOTAP does not induce NF-κB in DC, indicating a unique pathway of DC activation. Thus, DC activated by DOTAP/hE7 could be safely used for autologous adoptive transfer therapy.

Finally, the third clinical approach may be carried out using *ex vivo* preparation of human T cells directly activated by DOTAP/hE7. Our data provide the evidence that DOTAP interacts with human T cells, leading to the ERK phosphorylation in the T cells. Upon the treatment of DOTAP or DOTAP/hE7, the expanding T cell colonies were observed in the culture. However, only the cells treated with DOTAP/hE7 exhibited CTL capability with antigen specificity. Based on these results, a model is proposed in **Fig. 5.1**. The upper panel of the illustration shows antigen presentation by DC which is believed as a central dogma. DOTAP/E7 is taken up by DC via endocytosis. T cells are activated by mature DC through the interaction of peptide-MHC-TCR complex and costimulatory signals. The lower panel of the illustration proposes a possible antigen presentation process in the absence of DC. The detail mechanism how DOTAP delivers the antigen to T cells is unclear. Since T cells are not known as professional antigen presenting cells, it is questionable that T cells would present antigen the same way as APC (i.e. DC). The activated T cells express costimulatory molecules (CD80 and CD86) such that the event of antigen presentation may occur between two T cells followed by subsequent immune responses.

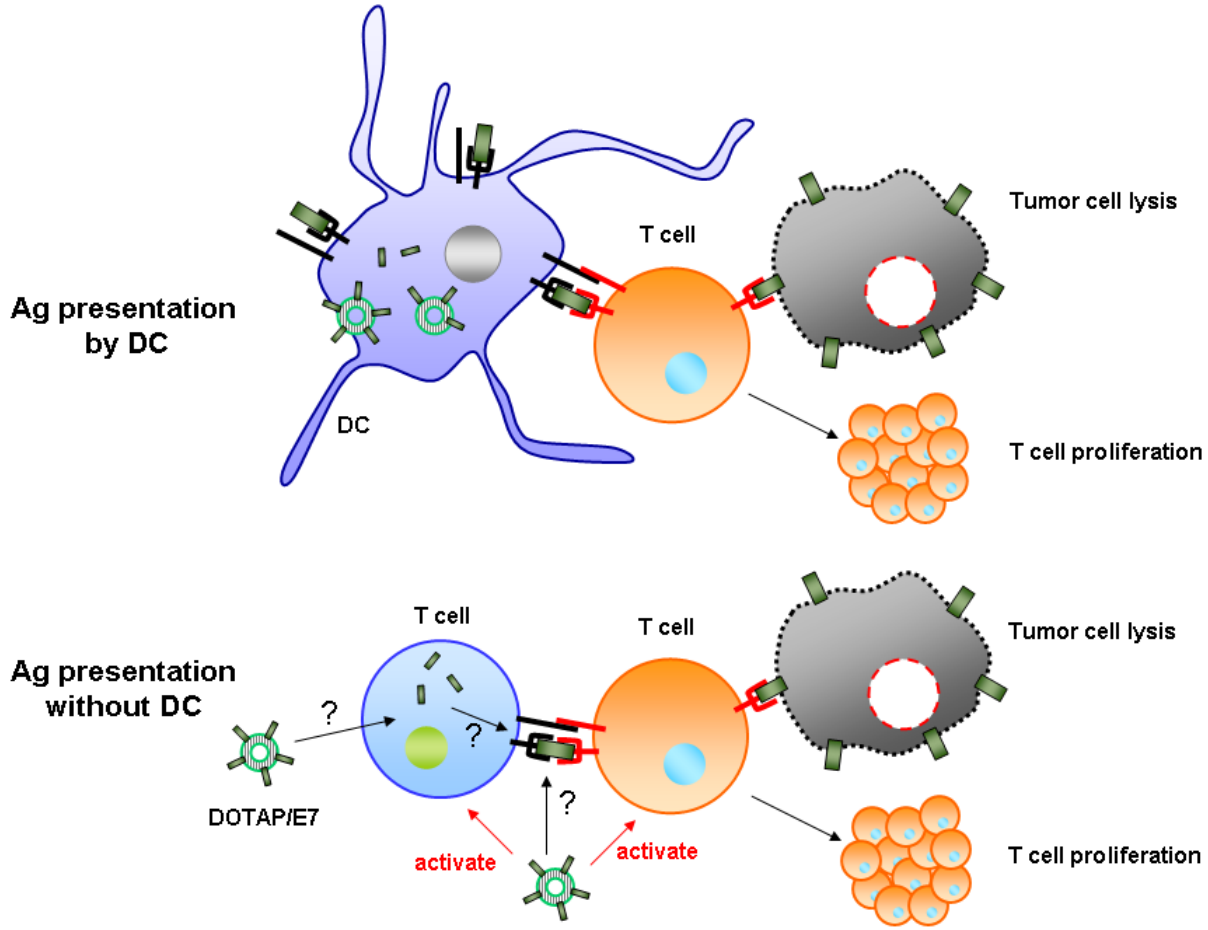


**Figure 5.1. A proposed model for antigen presentation and immune responses mediated by DOTAP/hE7 therapeutic vaccine.**

Despite many superior advantages of the DOTAP/E7 vaccine formulations described in the previous chapters, there are some issues that deserved more discussion. In **Fig. 2.4**, the high dose of DOTAP seems to be capable of stimulating CTL using a traditional CTL assay method which requires *in vitro* stimulation of responder T cells for 5 days in the presence of peptide. We cannot rule out the possibility that the true functionality of $CD8^+$ cells was overestimated after *in vitro* expansion of the subset cells. On the contrary, an *in vivo* CTL result (**Fig. 2.5A**) gave us a more trustworthy estimation of the functionality of $CD8^+$ T cells elicited in the mice. Our *in vivo* CTL results indicated that high dose of DOTAP does not generate efficient CTL *in vivo*.

Although DOTAP at high dose could generate a good NK response toward a non-specific killing, the effect contributed little to the anti-tumor activity. It is not clear at present the role of NK cells play in the overall anti-tumor activity induced by DOTAP/E7. Also, the *in vivo* CTL result shows that DOTAP/E7 vaccine was able to induce antigen-specific CTLs in the spleen, indicating E7-specific T cells present in DLN have migrated to the secondary lymphoid organ. It is believed that patrolling cells of the immune system provide continuously body-wide surveillance, spying out and eliminating tumor cells. However, we do not have information on the antigen-specificity of T cells at the tumor sites.

Our data in **Chapter 2** have indicated that the reduced anti-tumor activity of DOTAP/E7 at high dose lipid was associated with higher level of Tregs, probably owing to the excess ROS production and cell death in DC. Although the excess amount of ROS signal induces DC apoptosis, however, it is now known that ROS at low concentrations serve as an essential second messenger mediating cellular responses to many physiological stimuli [4]. DC treated with antioxidants are resistant to phenotypic and functional changes following stimulation with proinflammatory cytokines [5]. Following treatment with antioxidants, the levels of intracellular ROS are reduced, and the protein kinase RNA-regulated NF-κB, protein kinase C, and p38 MAPK pathways could not be activated following inflammatory agent stimulation [5]. Also ROS are shown in the DC interaction with T cells. For example, both T cells and DC elevate intracellular oxidation states upon Ag-specific interaction. Ebselen, an antioxidant, at 5–20 μM concentrations inhibits DC-induced proliferation and cytokine production by T cells as well as T cell-induced cytokine production by DC [6]. ROS also induce early phenotypic maturation of DC by up-regulating specific markers CD80, CD83, and CD86 and down-regulating mannose

receptor-mediated endocytosis [7]. Recently, cationic liposomes were reported to generate the ROS in macrophages leading to activation of the p38 pathway [8]. Therefore, cationic DOTAP liposome-induced ROS in antigen presenting cells plays a positive and essential role in the generation of immune response to protect the host.

Although immunization with the hE7 peptide (CTL epitope) resulted in a potent anti-E7 and tumor specific activity, immunization with the whole E7 protein might be more effective because there are multiple MHC class I and II restricted epitopes on the protein. The existence of highly diverse MHC I and II molecules among human populations also makes the whole E7 protein more attractive [9]. Instead of using the E7 peptide which encodes a single CTL epitope, the entire E7 protein formulated into cationic DOTAP liposome is an alternative strategy. The production of antibody response and the proliferation of $CD4^+$ T lymphocytes are the additional benefits of the protein antigen.

An interesting finding from an unpublished *in vitro* result using fluorescent NBD-DOTAP treated mouse spleen cells shows that $CD40^+$ B-cell population tends to uptake (bind to) a higher amount of DOTAP compared to other cell types (**Fig. 5.2**). The profile of NBD-DOTAP uptake by total spleen cells is shown in grey color (upper left). Despite the fact that NBD were taken up by both $CD3^+$ T (lower left, blue) and $CD40^+$ B cells (upper right, yellow), the intensity of NBD signal in B cells was 3-fold and 7-fold stronger than that in $CD4^+$ (green) and $CD8^+$ (red, lower right) T cells, respectively. This result implies that B cells may have more surface molecules that recognize/interact with DOTAP compared to other spleen cells.

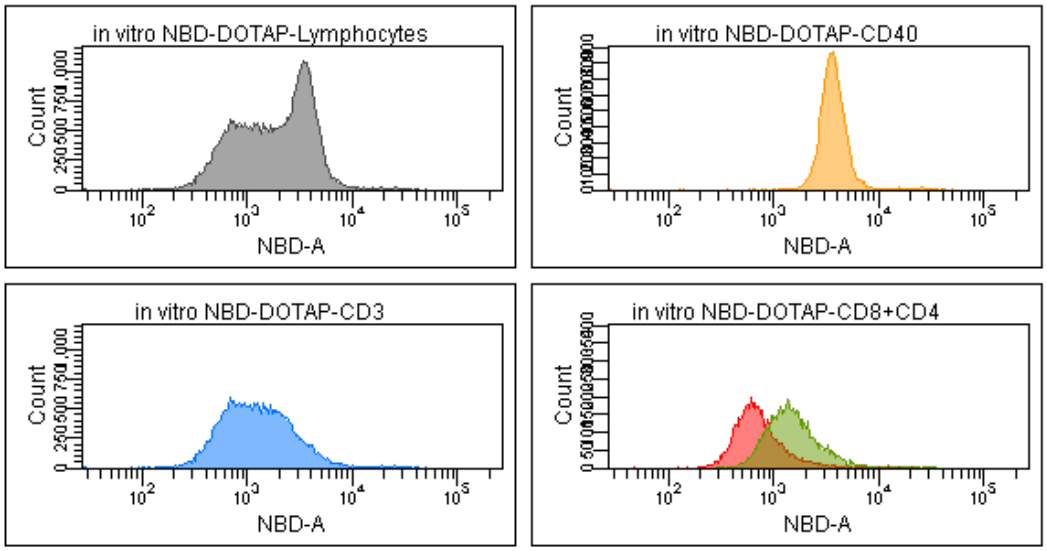


**Figure 5.2. *In vitro* NBD-DOTAP uptake by mouse spleen cells.**
Single cell suspension of mouse spleen cells were prepared and 50 μM DOTAP containing 1% fluorescent NBD-DOTAP was added into the culture. Spleen cells were harvested and label with cell surface marker prior to analysis by flow cytometry.

Furthermore, a separate experiment shown in **Fig. 5.3** that DOTAP can activate B cells, leading to expression of CD86 expression. The amounts of $CD19^+/CD40^+$ (blue dots) and $CD19^+/CD40^-$ (yellow dots) B cells were significantly increased after DOTAP stimulation *in vitro*. Cells gated within upper-right and lower-right quadrants were further analyzed for the expression of CD86 (shown in histogram). The data show that DOTAP induced the expression of $CD19^+$ molecule (a BCR co-receptor) as well as an activation marker (CD86) on B cells. Based on these preliminary data, we conclude DOTAP is efficient on activating B cells, thereby a B-cell targeted delivery by DOTAP can be proposed.

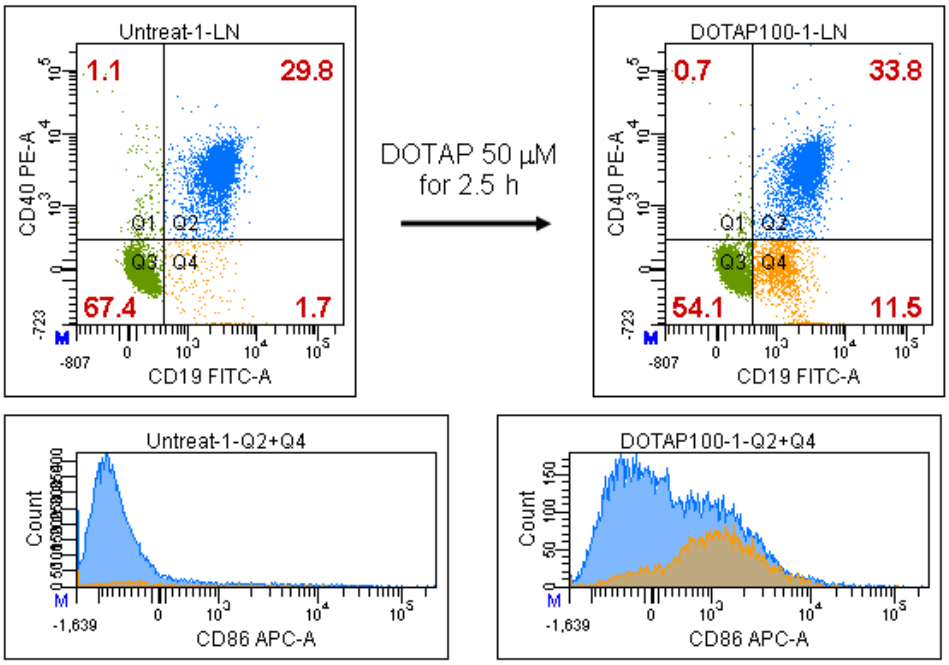


**Figure 5.3. B cells are activated by DOTAP stimulation *in vitro*.**
The dot plot and histogram on the left show the phenotype of untreated cells compared to that of the DOTAP treated cell on the right. The numbers shown on the dot plot represent the percentage of cell number for each quadrant.

The future directions of DOTAP/E7 vaccine project include the study of the interaction between DOTAP and other immune cells such as T and B cells. We have previously focused on the DOTAP-mediated signaling pathways in DC; however, results presented in this report show DOTAP also interact directly with other types of cell which deserved further investigation. We are also interested in discovering new cationic lipids which may exhibit an enhanced immune-modulatory effect compared to DOTAP.

It is also tempting to combine DOTAP/E7 vaccine with other active therapeutic strategies. Immunotherapy has been developed as an adjuvant therapy for human cancer for many years with limited success [10] [11]. One of the major reasons for failure is the fact that cancer patients

are frequently immuno-compromised, either due to the cancer growth itself or, more significantly, the use of various chemotherapeutic agents or radiation. Thus, it is important that any treatment prior to the immunotherapy does not suppress the immune system. Since one can use siRNA to silence specific target genes such that tumor apoptosis can be induced in the primary tumor without immune compromise, the subsequent immunotherapy should be more effective than if it is preceded by chemotherapy. Our lab has proposed an ideal method to induce primary tumor apoptosis and introduce cytokine at the same time via i.v. injection. It requires a specific tumor delivery of both plasmid DNA and apoptotic inducing agents without suppressing the immune system of the host (unpublished data).

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