INTERACTION OF CATIONIC LIPID VACCINES WITH CELLS OF THE ADAPTIVE IMMUNE SYSTEM

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When the cationic lipid DOTAP (1,2-dioleyltrimethylammoniumpropane) is used to encapsulate an antigenic peptide from the human papillomavirus E7 oncogene (E7), the resultant DOTAP/E7 particles act as a therapeutic vaccine to cause tumor regression through an antigen-specific immune response when the vaccine is injected into mice bearing E7-positive TC-1 tumors. Of critical importance, the DOTAP works as both a delivery vehicle and an adjuvant without induction of a pro-inflammatory cytokine response *in vivo*. It is hypothesized the antigen specific immune response is mediated by dendritic cells *in vivo*. To that end, the interaction of murine bone marrow-derived dendritic cells (BMDC) with the vaccine *in vitro* was investigated. When BMDC were incubated in the presence of DOTAP or the DOTAP/E7 vaccine, there was a dose and time-dependent upregulation of co-stimulatory molecule expression, indicating that BMDC were activated by the cationic lipid DOTAP. Further experiments indicated that BMDC were capable of internalizing DOTAP liposomes through many endocytic routes and the vaccine trafficked through the vacuolar pathway. An indirect method was used validate antigen presentation by BMDC, wherein the generation of antigen specific effector cells after incubation of CD8+ T lymphocytes, purified from the spleens of naïve mice, with fixed dendritic cells that had been activated by the vaccine, was examined. Not surprisingly, the DOTAP/E7 therapeutic vaccine was capable of initiating the generation of effector CD8+ T lymphocytes *in vitro* through a conventional mechanism, which requires

dendritic cell activation and presentation of the peptide antigen. Interestingly, it was found the same simple vaccine was capable of generating antigen specific effectors *in vitro* in the absence of antigen presenting cells in a novel pathway of effector generation, based on the expression of CD8 on the cell surface, T cell receptors that recognize a specific peptide antigen/MHC complex as well as an antigen-specific increase in IFN-γ production. Further studies revealed a possible mechanism for this novel pathway. Taken as a whole, these observations may lead to additional applications of DOTAP both *in vitro* and *in vivo* to modulate the immune response toward the correction of a variety of diseases.

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PREFACE

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1.0 INTRODUCTION

1.1 CELLS OF THE ADAPTIVE IMMUNE SYSTEM

The adaptive immune system responds to a specific inducing agent and mediates acquired immunity [1-3]. Additionally, the adaptive immune response is enhanced upon repeated stimulation, which is called immunological memory, a hallmark of the adaptive immune system [1-3]. The adaptive immune system has two components: humoral and cell-mediated immunity [1-3]. The humoral immune system is particularly important for response to soluble antigen whereas the cell-mediated immunity is essential for response to antigen that originates within a cell whereas cell-mediated immunity is conferred by antigen presenting and effector cells [1-3].

1.1.1 Dendritic cells are mediators of adaptive immunity

1.1.1.1 Dendritic cells are potent antigen presenting cells

Dendritic cells are professional antigen presenting cells that are extremely important for the induction of both humoral and cellular immune responses and are, therefore, crucial to the design of immunological therapies. To most effectively capitalize on their abilities, the biology of dendritic cells must be fully understood.

Immature dendritic cells are peripheral sentinels that have high endocytic ability [1, 4]. They have low levels of surface MHCI and II and costimulatory molecules such as CD80 and

CD86 [1, 4]. Mature dendritic cells migrate to the local lymph node where they present antigen on upregulated MHCI and II molecules to T cells and activate those cells through upregulated costimulatory molecules on their surface [1, 4].

Dendritic cells are professional antigen presenting cells that can efficiently prime and activate both naïve and memory T cells [5]. LPS, DNA with CpG motifs, immune complexes, apoptotic cells, dsRNA, prostaglandins and proinflammatory cytokines will activate and induce maturation of dendritic cells [5, 6] through Toll-like receptors (TLR), which recognize features common to pathogens. Expression of co-stimulatory molecules such as CD80 and CD86 is upregulated upon DC activation [5-7] and this increased expression is a common way to define dendritic cell activation [8]. Once activated, dendritic cells have downregulated endocytic activity [1, 4-7] and disassemble their actin-based cytoskeleton [4].

Contrary to accepted dogma, it has been shown that dendritic cells immediately up-regulate endocytosis (macropinocytosis) through actin cytoskeleton rearrangements for enhanced antigen capture and presentation [9]. A proposed mechanism is the disassembly of actin-rich podosomes to fuel actin-dependent endocytosis [9].

1.1.1.2 Internalization pathways of dendritic cells

Immature DCs internalize antigen by receptor and non-receptor-mediated endocytic pathways including pinocytosis, macropinocytosis and phagocytosis [5, 6, 10]. Phagocytosis and pinocytosis are distinguished based on the size of the particle taken up and the requirement of actin polymerization [11]. Phagocytosis internalizes large particles (>200nm) and involves actin polymerization whereas pinocytosis internalizes small particles (<200nm) and does not involve actin polymerization [11]. Both clathrin- and caveolar-mediated endocytosis are dynamin dependent [10, 11] and are involved in cell signaling [11].

There is speculation that dendritic cells might have unique caveolar-mediated endocytosis pathways [12]. Caveolae were described in endothelial cells as smooth invaginations of the plasma membrane [13]. The caveolae have common identification markers including ganglioside G_{M1} and caveolin [14]. Caveolin can migrate directly from caveolae to the ER (endoplasmic reticulum) and is responsible for transporting newly synthesized cholesterol from the ER to caveolae [15]. Caveolar endocytosis internalizes components of lipid rafts on the cell surface and is defined by sensitivity to cholesterol depletion [10]. Cholesterol binding agents such as MßCD (Methyl-β-cyclodextrin) [12, 13], lysenin, and filipin [14] will inhibit caveolar-mediated endocytosis. Depletion of membrane cholesterol prevents cholera toxin (CTX) entry into cells [13] since ganglioside G_{M1} is the receptor for CTX [14]. It has also been shown that 10μg/ml progesterone blocks cholesterol movement [15] and, interestingly, that caveolin 1, the main protein component of caveolae, slows or inhibits endocytosis [10].

Other inhibitors of endocytosis include a 4°C temperature block to prevent internalization from the plasma membrane, a 20°C temperature block of endosomal trafficking [13], brefeldin A to block Golgi trafficking [13] and nocodazole to block trafficking to caveosomes [13]. As studied in endothelial cells, caveolin is internalized from the membrane to form vesicular caveolae, which then move to caveosomes.

1.1.2 CD8+ T lymphocytes are effectors

T lymphocytes are members of the adaptive immune system that develop in the thymus from bone marrow precursors [1-3]. The T cells develop the T cell receptor (TCR) and differentiate into one of two peripheral subsets [1-3]. This subset comprises about 25% of the circulating lymphocytes, which represent 20% of the total blood leucocytes [1-3]. T cells bearing the CD4

marker are commonly known as helper T cells whereas CD8+ cells are the cytotoxic T (Tc) cells [1-3].

Cytotoxic T cells are capable of destroying virally infected cells by means of recognition of viral antigen presented by the MHCI molecule [1-3]. Tc cells recognize the antigen in the context of the MHCI molecule via the TCR. The TCR is an integral membrane protein that exists on all mature T cells that specifically recognizes antigen associated with MHC molecules [1-3]. The TCR is made up of two components: two MHC/antigen binding dimers and the invariant CD3 complex, which is required for cellular activation [1-3].

In order to fully activate a T cell, three requirements must be met [1-3]. First, the antigenic peptide must be presented by an MHC molecule [1-3]. Second, there must be a co-stimulatory signal, which is conferred by the presence of co-stimulatory molecules on the surface of the antigen presenting cell [1-3]. Third, the cell must receive signaling from specific cytokines [1-3]. An absence of any one of these signals will not cause activation and can lead to anergy (unresponsiveness) or death [1-3].

Activated Tc cells are the cellular effectors of the adaptive immune system [1-3]. Upon recognition of appropriate target cells, the Tc cells release cytotoxic granules at the cellular junction leading to apoptosis of the target cell [1-3]. The granules contain perforin, which forms channels on the cell membrane through which the also released granzymes enter [1-3]. The granzymes are serine proteases that activate enzymes responsible for DNA degradation and apoptosis [1-3]. Activated T cells also release IFN-γ, which limits the spread of viral infections by binding the receptors of nearby cells, causing them to produce antiviral proteins [1-3].

1.1.3 Cancer cells as targets

The immunology of a tumor plays a critical role in the potential for the immune system to respond to that tumor. Tumors are a difficult target as most, if not all, antigens expressed by the tumor cells are self antigens and specific 'self' T cells have already been deleted in the thymus. Additionally, the tumor cells can escape rejection in many ways, avoiding 'immune surveillance', which is the ability of the immune system to detect and destroy tumor cells.

There exist six classes of tumor rejection antigens (TRA), which are antigens recognized by CTL from clinical patients [1, 2]. The six classes are tumor-specific mutated oncogene or tumor suppressor, germ cell, differentiation, abnormal gene expression, abnormal post-translational modification, oncoviral protein [1, 2]. In the context of this study, the sixth category of TRA (oncoviral protein) is most relevant. These proteins may have a critical role in the oncogenic process and due to their foreign source, they may be capable of eliciting a T cell response [1, 2]. The human papillomavirus 16 E6 and E7 proteins are members of this class.

Although the oncoviral protein can stimulate an anti-tumor response *in vitro* and *in vivo*, it is rare for an established tumor to be spontaneously rejected. Tumors have distinct evasion mechanisms that allow them to survive despite a healthy immune system.

Tumors can escape immune recognition because of low immunogenicity, presentation of self antigen, antigenic modulation, tumor-induced immune suppression and tumor-induced privileged sites [1, 2]. Weakly immunogenic tumor cells have low levels of surface MHCI (and MHCI/peptide complex) [1, 2]. Additionally, there can be low levels of adhesion and co-stimulatory molecules on weakly immunogenic tumor cells [1, 2]. If the tumor cells display self antigen in the absence of co-stimulation, tolerant T cells will result [1, 2].

1.2 IMMUNITY BY VACCINATION

1.2.1 Peptide cancer vaccines

Peptide vaccines are one of the strategies used to overcome the evasion methods of the tumor toward induction of a potent anti-tumor response *in vivo* [16]. Peptide epitopes for cytotoxic T cells are not as immunogenic as full length protein and thus require additional components to enhance vaccine efficacy [16]. Several strategies are used to increase the efficiency of the peptide vaccines: epitope enhancement, inclusion of helper T cell epitopes, incorporation of immunostimulatory molecules, blockade of negative regulators and delivery of peptide on dendritic cells [16]. Coating the dendritic cells or other antigen presenting cells with peptide is used as a type of autologous cellular vaccine, which is a beneficial strategy because it allows the efficient, natural process of T cell activation to proceed [16]. Additionally, peptide vaccines also have the advantages of chemical stability and definition [17].

1.2.1.1 Cervical cancer as a model system

Cervical cancer is the second most common cause of cancer in women with 500,000 new cases diagnosed per year. Human papillomavirus (HPV) is the causative agent in at least 95% of those cases [18]. The incidence rate for cervical cancer is 10 in 100,000 women in developed countries and is 4 times higher in developing countries [18]. Of the many subtypes of HPV, subtypes 16 and 18 are most implicated in cervical cancer. Subtype 16 is most common, with 50% of all cases HPV16-positive [19]. HPV types 16 and 18 are the most dominant types found in cancers and their oncoproteins, E6 and E7, are powerful disrupters of the cell cycle through

interaction with E2F and Retinoblastoma (Rb) protein. HPV16 E7 is continuously expressed in cancer cells and pre-cancerous lesions and is a unique antigen for cervical tumors [20].

The TC-1 cell line, a transformed murine cell line that constitutively expresses HPV16 E7, is used in this study because it causes histocompatible E7-positive tumors in C57BL/6 mice in a murine model of human cervical cancer. Histocompatibility is essential to the study of antigen specific cell-mediated immunity. The C57BL/6 mice express H2D[b] and H-2K[b] and the E7 peptide (RAHYNIVTF) is the immunodominant H2D[b]-restricted epitope of the E7 oncoprotein. The control peptide used in this study is NP (ASNENMETM), the H2D[b]-restricted immunodominant epitope of the influenza strain A/PR/8/34 nucleoprotein.

1.2.1.2 Therapeutic cancer vaccines

Cationic lipid-based therapeutic cancer vaccines have been developed in the last decade to capitalize on the unique properties of cationic lipids while overcoming the obstacles of creating an effective immune response to an established tumor. The LPD-based vaccine has several features that make it ideally suited to deliver tumor-associated antigen in the context of vaccination [21]. When LPD are administered intravenously, the unmethylated (CpG) DNA generates a systemic Th1-like innate immune response, including induction of the Th1 cytokines IFN-g, TNF-a and IL-12, which are correlated with tumoristatic effects [21, 22]. When administered to mice, the LPD/E7 vaccine is therapeutic, causing regression of established TC-1 tumors [23].

Moreover, it was found that all components of the LPD/E7 vaccine except for the DOTAP lipid and the E7 peptide could be removed without loss of function *in vivo* [24]. At the optimal lipid dose, DOTAP/E7 caused TC-1 tumor regression and was as effective as the LPD/E7 vaccine at the same lipid dose [24]. This observation suggests the DOTAP cationic

lipid provides adjuvant activity in the absence of plasmid DNA and the unmethylated CpG motifs [24]. Furthermore, the DOTAP/E7 vaccine induced migration of activated dendritic cells to the draining lymph node and generated functional antigen-specific CD8+ T lymphocytes [24].

These results provide a framework on which this study is based. The goal of this project is to verify and recapitulate *in vitro* the hypothesis that the cationic lipid vaccines interact with dendritic cells *in vivo* to generate an antigen specific anti-tumor response.

1.2.2 Cationic Lipid Vaccines

Liposome-protamine-DNA (LPD) nanoparticles were first described as a nonviral vector for gene therapy applications [25]. The LPD has a net positive charge resulting from the use of the cationic lipid DOTAP (1,2-dioleyltrimethylammoniumpropane) (Figure 1). The positively charged LPD particles are able to transfect many cell types because of the negative cell surface charge [17]. The LPD particles afford stability to the DNA as it is protected from serum nucleases in the condensed, entrapped state [17]. The LPD particles were originally designed as a liposome-based gene delivery vehicle but are now being exploited as peptide vaccine carriers [17]. Advantages of nonviral vaccines include safety, cost-effectiveness, ease large scale production and long term stability.

1.2.2.1 Cationic lipid vaccine structure

When protamine-condensed plasmid DNA is added drop wise to cationic liposomes, the liposomes spontaneously rearrange to encapsulate the DNA in the internal, aqueous environment and form liposome-protamine-DNA (LPD) nanoparticles. The LPD are self-assembling homogenous particles with a diameter slightly less than 100nm [25]. The simpler DOTAP/E7

vaccine was formulated (as described in the Materials and Methods section) with the cationic lipid DOTAP and imaged using a transmission electron microscope with negative staining (Figure 2). For fluorescent tracking studies, a fluorescent DOTAP analogue (NBD-DOTAP) (Figure 1) is routinely used.

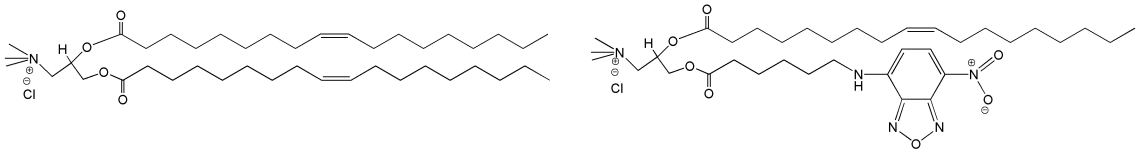


Figure 1. Chemical structures of cationic lipids

DOTAP (left) and fluorescent analogue, NBD-DOTAP (right)

Representative images (Figure 2) confirm that a bilayer, which is indicative of liposomal structure, is not present in the DOTAP/E7 vaccine particles. It appears the vaccine consists of amorphous complexes of lipid and peptide with no discernable physical structure. The vaccine particles have a size of approximately 200nm or less in diameter.

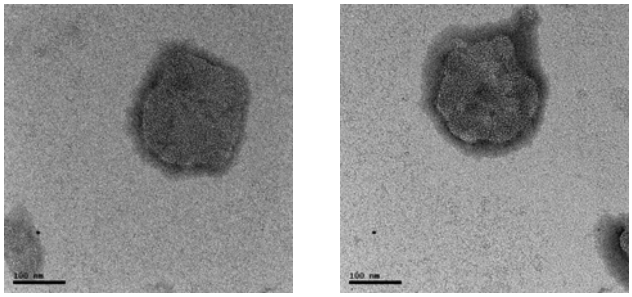


Figure 2. Electron micrographs of DOTAP/E7 cationic lipid vaccine

Two unique transmission electron micrographs of DOTAP/E7 vaccine particles. Scale bars equal 100nm.

1.2.2.2 Adjuvant properties

The role of cationic lipid in dendritic cell activation is currently being investigated, but it is known that unmethylated CpG motifs of the plasmid DNA in the LPD/E7 vaccine can activate dendritic cells via TLR9. This property makes the LPD a unique vaccine carrier and natural adjuvant, which stimulates both humoral and cellular immune responses [21].

Knowing the simple vaccine consisting only of lipid and peptide can generate an immune response, it is believed the DOTAP lipid component acts as both a protective delivery vehicle for the peptide as well as an adjuvant, activating the immune system. To determine if the DOTAP lipid adjuvant elicits a pro-inflammatory cytokine response in vivo, naïve C57BL/6 mice were injected subcutaneously with DOTAP, DOTAP/E7 vaccine or left untreated (75nmole DOTAP /10μg E7 /150ul 5% dextrose per mouse). Blood and local (inguinal) lymph nodes were collected from 4 mice per time point and analyzed for TNF-α and IL-12 by ELISA. As indicated in Table 1, the pro-inflammatory cytokines are induced neither systemically nor locally in the

lymph nodes. Specifically, the circulating TNF-α levels were below the minimum detection limit and all other groups were not statistically different from that of untreated (0 hour) mice.

Perhaps counter-intuitively, the induction of pro-inflammatory cytokines is not necessarily a beneficial adjuvant effect. Indeed, it is necessary to stimulate the immune system in order to elicit an adaptive immune response, but the induction of pro-inflammatory cytokine can have devastating effects in a healthy body. The production of pro-inflammatory cytokines in response to vaccine administration is deemed a negative quality by the FDA and has surely caused the failure of many vaccines during clinical trials because of safety concerns.

There is a recent publication by Ross, *et al.* that discusses this same idea. Adjuvants need not produce serum TNF-α to be effective [26]. In this study, lipopolysaccharide (LPS), a potent inducer of pro-inflammatory cytokines *in vivo*, was administered subcutaneously to guinea pigs. These animals developed elevated temperatures but had no detectable TNF-α in the blood circulation. The authors speculate the adaptive immune system causes the fever even in the absence of circulating TNF-α. Therefore, it is reasonable to assume the DOTAP/E7 vaccine will not produce serum TNF-α since it is also administered subcutaneously.

Table 1. In vivo cytokine levels after subcutaneous vaccine administration
In vivo pro-inflammatory cytokine levels after subcutaneous vaccine administration. DOTAP and DOTAP/E7 were not significantly different from untreated or each other at matched time points ($p>0.1$).

IL-12				
	DOTAP		DOTAP/E7	
Time (hours)	serum (pg/ml)	lymph nodes (pg/LN)	serum (pg/ml)	lymph nodes (pg/LN)
0	111.3	40.8	111.3	40.8
2	84.9*	33.0*	41.4*	62.8*
4	51.1*	36.9*	144.2*	52.7*
6	136.3*	21.6*	121.0*	31.4*

* $p>0.1$ compared to untreated and time matched control

TNF-α				
	DOTAP		DOTAP/E7	
Time (hours)	serum (pg/ml)	lymph nodes (pg/LN)	serum (pg/ml)	lymph nodes (pg/LN)
0	ND†	8.0	ND†	8.0
2	ND†	13.1*	ND†	13.1*
4	ND†	10.1*	ND†	2.8*
6	ND†	8.3*	ND†	6.1*

†TNF-α concentrations were below minimum detection limit
* $p>0.1$ compared to untreated and time matched control

2.0 INTERACTION OF LPD VACCINE WITH DENDRITIC CELLS

2.1 INTRODUCTION

Dendritic cells are mediators of adaptive immunity, allowing the body to specifically recognize and eliminate non-self antigens. It has previously been demonstrated that our cationic lipid LPD based vaccine formulation (LPD/E7) can stimulate an anti-tumor response in a mouse model [23]. This occurs through the initiation of an immune response via TLR activation [23]. Coupled with delivery of a tumor-specific peptide, the immune system eliminates the tumor cells bearing the peptide in order to cause tumor regression [23].

Knowledge obtained through these studies of basic dendritic cell biology and their interaction with nonviral vectors can be used to enhance several fields of research, including of immunology, cancer therapy, DNA vaccines and other nonviral vaccines, including LPD. These discoveries will allow the modulation of vaccine formulation to enhance the current vaccine and develop new formulations for alternative applications.

2.2 MATERIALS AND METHODS

2.2.1 Reagents

1,2-Dioleoyl-3-Trimethylammonium-Propane (DOTAP) and 1-oleoyl-2-[6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]hexanoyl]-3-trimethylammonium propane (18:1-06:0 NBD DOTAP) were purchased from Avanti Polar Lipids (Figure 1). Lipopolysaccharide and protamine sulfate were purchased from Sigma Chemical Company. RPMI-1640, non-essential amino acids, antibiotic/antimycotic and PBS were purchased from Gibco Chemical Company and GM-CSF and IL-4 mouse cytokines were purchased from R&D Systems. All antibodies were purchased from Becton Dickinson unless otherwise specified.

2.2.2 Cell culture

The DC2.4 dendritic cell line was maintained in RPMI-1640 growth medium supplemented with 10% fetal bovine serum (FBS), 100U/ml penicillin and 100mg/ml streptomycin at 37°C and 5% CO_2. Primary dendritic cells were differentiated from murine bone marrow precursors in RPMI-1640 supplemented with 10% FBS, NEAA, antibiotic, antimycotic, GM-CSF and IL-4 for 6 days at 37°C and 5% CO_2 in a protocol adapted by the author from Inaba, *et al* [27].

2.2.3 Preparation of LPD nanoparticle vaccine

The DOTAP (1,2-dioleyltrimethylammonium propane) cationic liposomes are prepared by thin film hydration followed by sonication and extrusion to 0.1μm. For fluorescent liposomes, NBD-DOTAP was mixed with DOTAP in chloroform at a 1/100 molar ratio prior to solvent evaporation. To prepare DOTAP/E7 particles, the liposomes were hydrated in a peptide solution and unencapsulated E7 was not removed after extrusion. To prepare LPD nanoparticles, immediately prior to use, the protamine-condensed plasmid DNA was added to the liposomes dropwise at a 9.0:0.6:1.0 weight ratio (DOTAP : salmon sperm protamine sulfate : plasmid DNA). To create fluorescent LPDs for flow cytometery or microscopy experiments, fluorescent-labeled DNA was mixed with unlabeled DNA at a 1:9 or 1:1 w/w ratio, respectively.

2.2.4 Treatment conditions

Pre-stimulation and treatment concentrations were 75μM DOTAP liposome, 6.25μg/ml DNA and 0.1μg/ml LPS (lipopolysaccharide) unless otherwise indicated. The LPD concentration used yielded working concentrations of DOTAP and DNA equal to controls.

2.2.5 Flow cytometry

Cells were seeded in 12 well plates ($1.5x10^6$/2ml/well) and allowed to attach for 2 hours before treatment with or without LPS for 16-20 hours. After LPS activation, cells were washed and indicated treatments were added for 1 hour before washing and staining for flow cytometry, if necessary.

2.2.6 Microscopy

For widefield fluorescence microscopy, $1x10^5$ cells were seeded in 500µl media per well in 8 well chamber slides. Samples were activated with or without LPS overnight and treated for 1 hour as indicated. Cells were stained with Trypan Blue to quench extracellular fluorescence, fixed with 2% paraformaldehyde for 30 minutes at 4°C and mounted for viewing at 40X. For confocal microscopy, $1x10^5$ cells were seeded per coverslip in 1 ml media per well of a 24 well plate. The cells were incubated overnight with or without LPS activation followed by treatment with LPD for 1 hour at 37°C. After washing twice with phosphate buffered saline (PBS), cells were fixed and permeabilized in methanol for 1 minute. Cells were washed twice in PBS and incubated with primary antibody (Ab) for 1 hour at room temperature. Cells were washed twice in PBS and incubated for 1 hour at room temperature with a secondary Ab conjugated to FITC (fluorescein isothiocyanate). Cells were washed twice with PBS, mounted onto slides and viewed at 60X with a confocal microscope.

2.3 RESULTS

Bone marrow-derived dendritic cells had morphology typical of dendritic cells (Figure 3) and were positive for CD11c, a surface marker for dendritic cells. For all experiments, cells were collected on day 6. The day 6 cells have low CD11c levels, indicative of immature dendritic cells (Figure 4). After an additional 24 hours in culture, the BMDC had high levels of CD11c (mature DC) and were only activated (high surface levels of CD86) when exposed to a powerful activator, LPS (Figure 4). Otherwise, the cells were considered to be "resting" as culture conditions alone did not activate these cells. Taken together, the cultured BMDC are morphologically and phenotypically consistent with high quality dendritic cells.

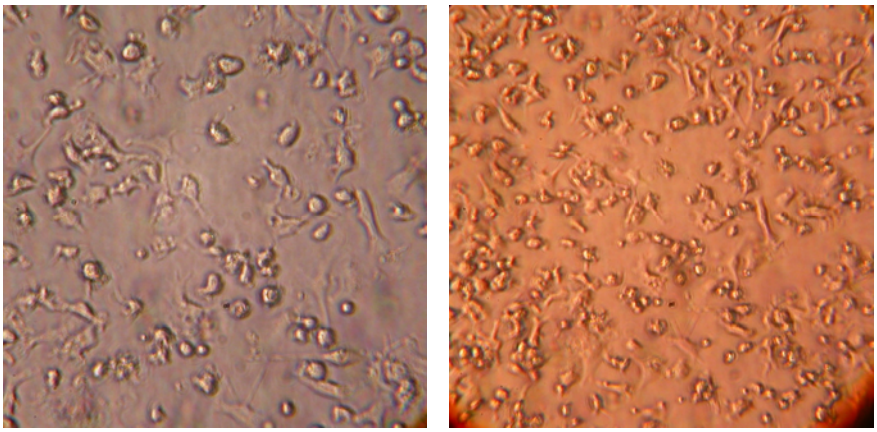


Figure 3. Photographs of bone marrow-derived dendritic cells in culture

Loosely adherent cells were collected on day 6, transferred to a new flask and incubated an additional 24 hours before microscopic analysis at 40X (left) and 25X (right) magnification.

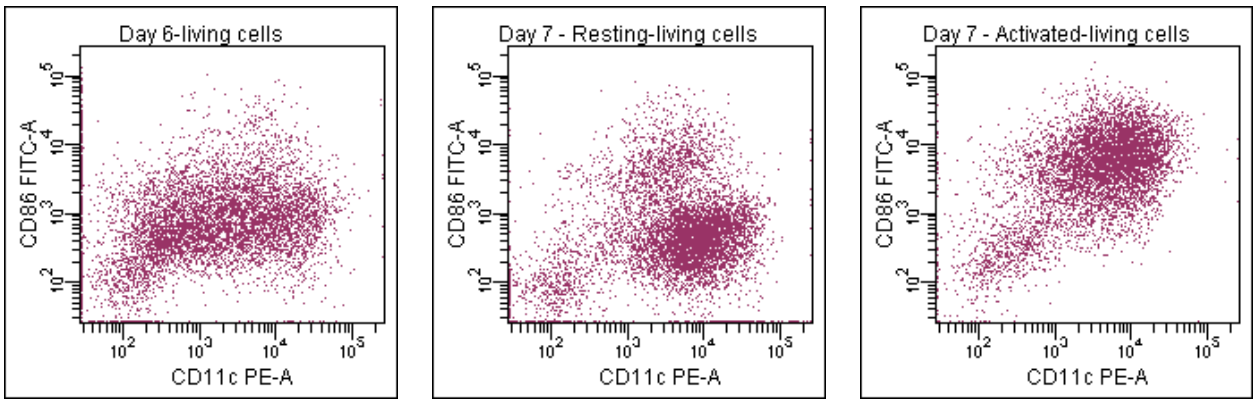


Figure 4. Characterization of BMDC in culture

Loosely adherent cells were collected on day 6 and incubated an additional 24 hours in the presence (activated) or absence (resting) of 0.1 µg/ml LPS. Cells were stained with α-CD11c-PE (DC-specific) and α-CD86-FITC (activation) and analyzed by flow cytometry.

DC2.4 cells, an immortal murine dendritic cell line, have been used as a model for dendritic cells, but the argument remains that they are dissimilar from primary dendritic cells. Both DC2.4 cells and BMDC were used throughout this study. As indicated in Figure 5, the two sources of murine dendritic cells, DC2.4 cell line and BMDC, yield different levels of activation upon stimulation, as analyzed for cell surface marker expression by flow cytometry. It appears that BMDC are much more sensitive to stimulation. Therefore, if the DC2.4 cells are highly activated, we can assume that the BMDC would also be.

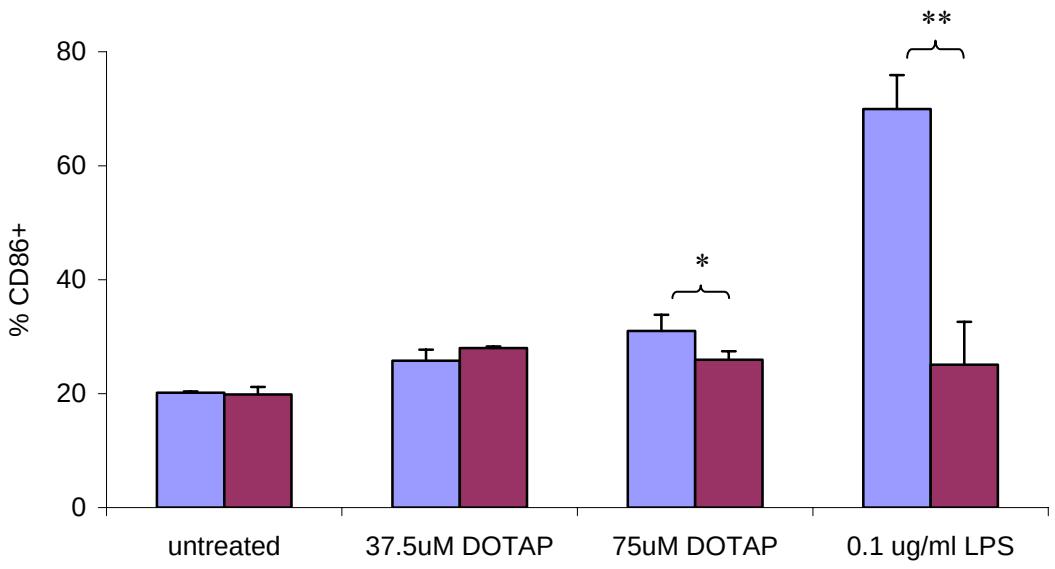


Figure 5. Dendritic cell source affects activation potential

DC2.4 (red) or bone marrow-derived dendritic cells (blue) were incubated in the presence of DOTAP, LPS or left untreated for 24 hours at which time the cells were stained for CD86 and analyzed by flow cytometry. Data was pooled from 3 experiments and t-test performed (*p=0.08, **p=0.02).

2.3.1 Effect of dendritic cell activation on internalization of LPD

2.3.1.1 Activation of dendritic cells

To first analyze activation, DC2.4 cells were stimulated with liposomes, LPD or LPS overnight and stained with α-CD80-PE for analysis by flow cytometry. As shown in Figure 6, all three treatments activated DC2.4 cells relative to the untreated control. As indicated on the histogram, overnight treatment with LPD activates DC2.4 cells. Moreover, LPD treatment appears to cause more activation than the positive control, LPS. To follow up on this observation, DC2.4 cells were treated overnight with LPD or LPS, followed by treatment for 1 hour with LPD and then stained with α-CD80-PE. As shown in the histogram (Figure 7), overnight treatment with LPD

fully activates DC2.4 cells. Treatment with additional LPD particles does not increase the activation profile for either LPD or LPS overnight activation. Once again, it appears that LPD particles are better activators of DC2.4 cells than is LPS.

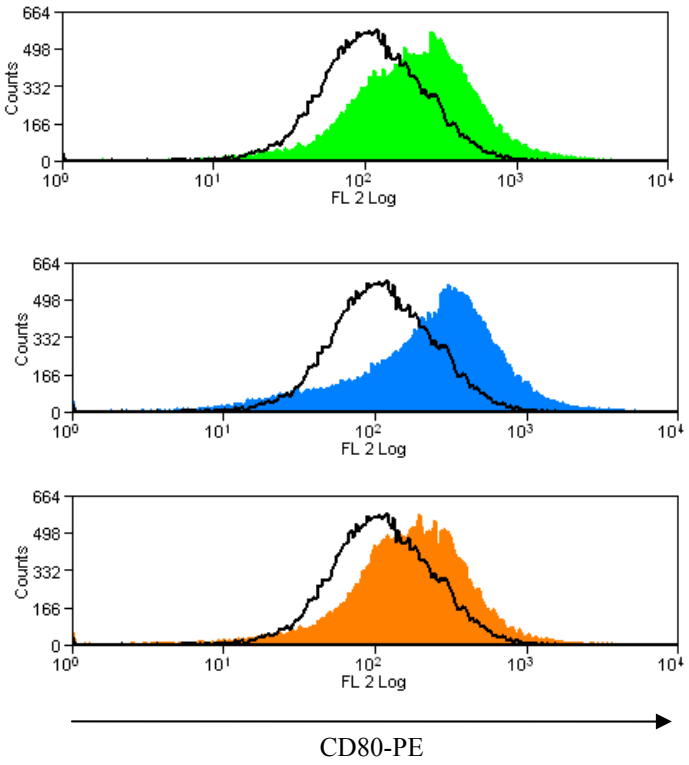


Figure 6. Overnight treatment with LPD activates DC2.4 cells.

Overnight treatment with LPD activates DC2.4 cells. DC2.4 cells were untreated (black line) or stimulated with liposomes (green), LPD (blue) or LPS (orange) overnight. Cells were washed, stained with α-CD80-PE and analyzed by flow cytometry, gated for living cells.

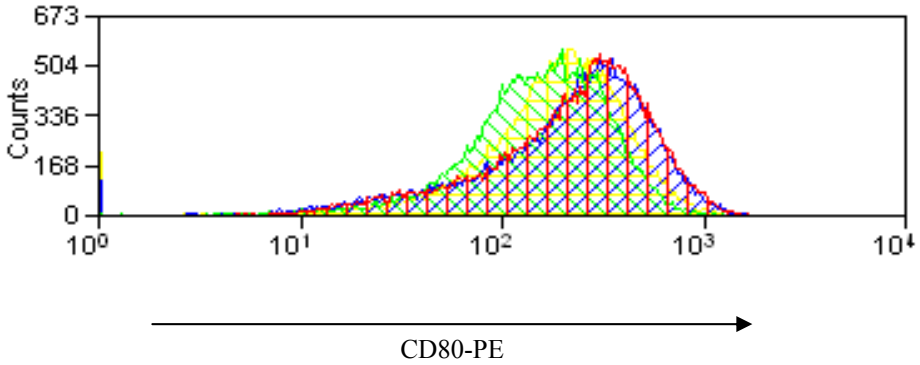


Figure 7. Overnight treatment with LPD fully activates DC2.4 cells.

Overnight treatment with LPD fully activates DC2.4 cells. DC2.4 cells were treated overnight with LPD (red, blue) or LPS (green, yellow), followed by no treatment or a 1 hour LPD treatment, respectively. Cells were washed, stained with α-CD80-PE and analyzed by flow cytometry, gated for living cells.

2.3.1.2 Internalization by resting dendritic cells

Knowing that DC2.4 cells are activated by LPD nanoparticles, it was tested whether these particles are internalized by the dendritic cells or merely activate at the surface. Immature DC2.4 cells were incubated for one hour with fluorescein labeled DNA or LPD prepared with fluorescein labeled DNA. Flow cytometry was used to analyze the amount of green fluorescence that was internalized by the cells. The amount of DNA used per sample remained constant, as did the ratio of fluorescent to non-fluorescent DNA. Figure 8 clearly shows that DC2.4 cells can internalize the fluorescent LPD nanoparticles, but do not take up much, if any, fluorescent labeled free DNA. Fluorescent microscopy was used to further analyze this observation. Resting DC2.4 or primary BMDC dendritic cells were treated for 1 hour with FAM (fluorescein)-labeled DNA or LPD prepared with FAM-labeled DNA. The results in Figure 9 are consistent with results obtained earlier. Both DC2.4 cells and BMDC can internalize LPD nanoparticles, but not free DNA.

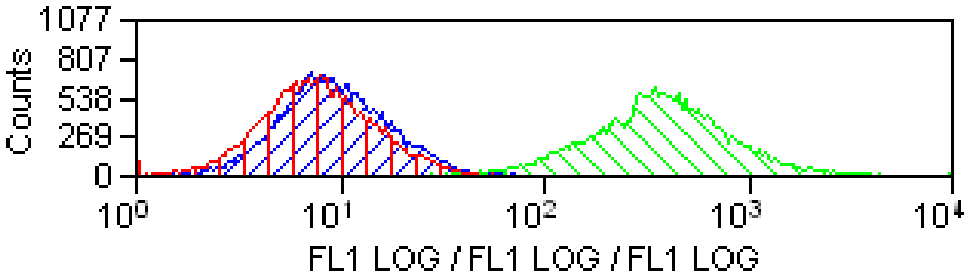


Figure 8. DC2.4 cells can internalize LPD but not free DNA.

DC2.4 cells can internalize LPD but not free DNA. Resting DC2.4 cells were untreated (red) or treated for 1 hour with fluorescein labeled DNA (blue) or LPD prepared with fluorescein labeled DNA (green). Cells were washed and analyzed by flow cytometry, gated for living cells.

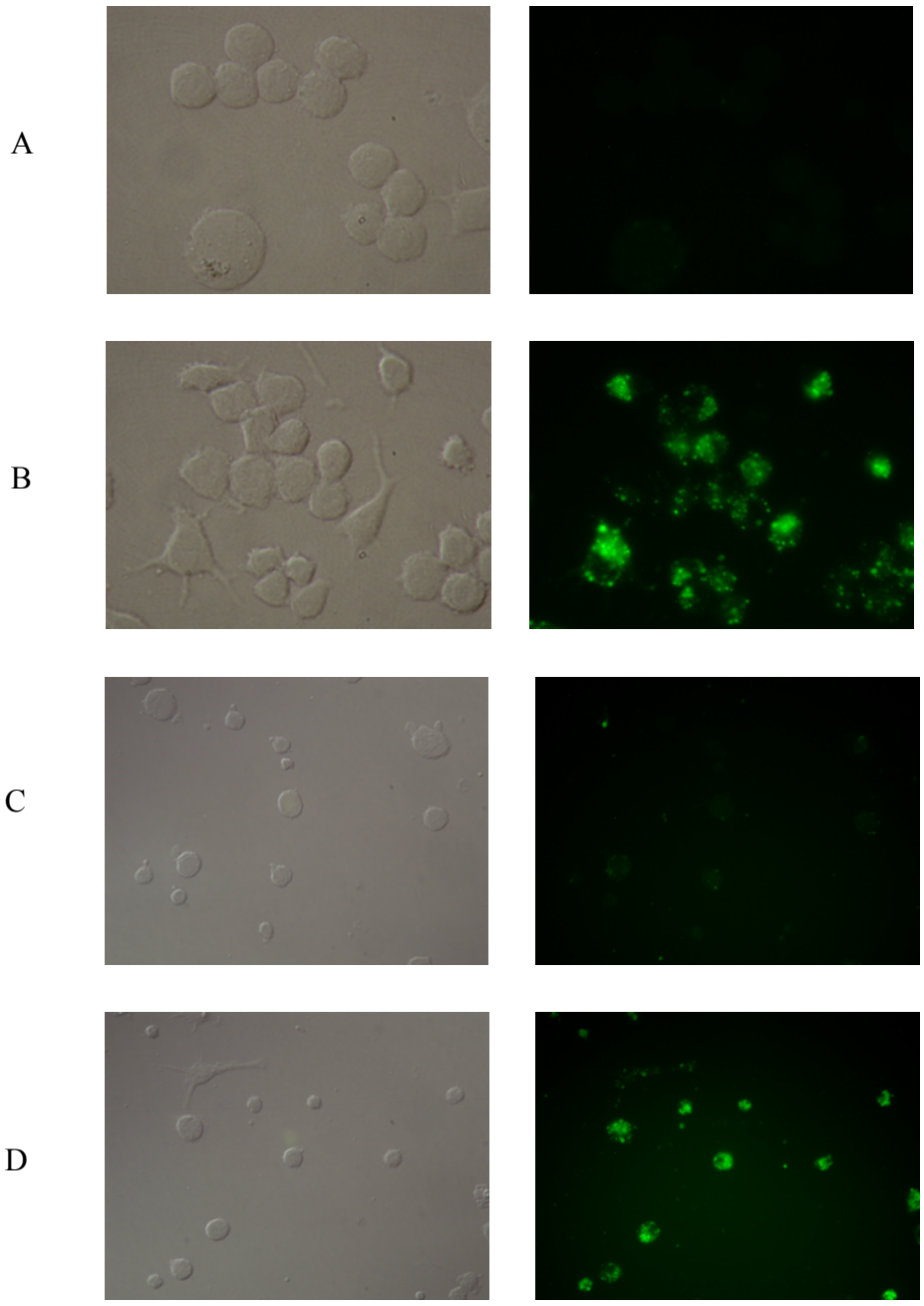


Figure 9. DC2.4 cells and primary dendritic cells can internalize LPD but not free DNA.

Resting DC2.4 (A,B) or primary BMDC dendritic cells (C,D) were treated for 1 hour with fluorescein-labeled DNA only (A,C) or LPD prepared with fluorescein-labeled DNA (B,D). Cells were washed and viewed at 40X magnification with a wide-field fluorescent microscope.

2.3.1.3 Internalization by activated dendritic cells

Dendritic cells were next tested for their ability to internalize LPD after activation. DC2.4 cells were activated overnight with LPS, which has already been shown to fully activate the dendritic cells. Cells were the treated for 1 hour with fluorescein labeled DNA or LPD prepared with fluorescein labeled DNA. Cells were washed, stained with α-CD80-PE and activated (CD80+) cells were analyzed for fluorescein (DNA internalization). The histogram in Figure 10 shows that activated DC2.4 cells can also internalize the fluorescent LPD particles but not free DNA. There is a slight shift in fluorescence for the free DNA, which could indicate slightly elevated internalization of free DNA by activated dendritic cells. Using fluorescent microscopy to again verify the flow cytometry results, DC2.4 or BMDC were activated overnight with LPS then treated for 1 hour with FAM-DNA or LPD prepared with FAM-DNA. The images in Figure 11 once again support the observation by flow cytometry, that activated dendritic cells internalize LPD but not free DNA.

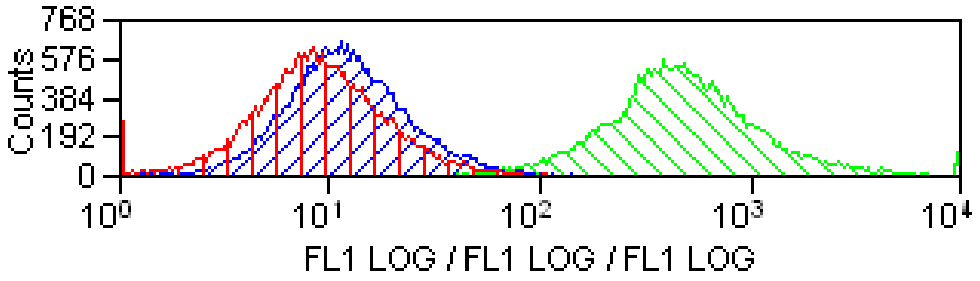


Figure 10. Activated DC2.4 cells can internalize LPD but not free DNA

Activated DC2.4 cells can internalize LPD but not free DNA. DC2.4 cells were activated overnight with LPS, followed by no treatment (red) or a 1 hour treatment with fluorescein labeled DNA (blue) or LPD prepared with fluorescein labeled DNA (green). Cells were washed, stained with α-CD80-PE and activated (CD80+) cells were analyzed for fluorescein.

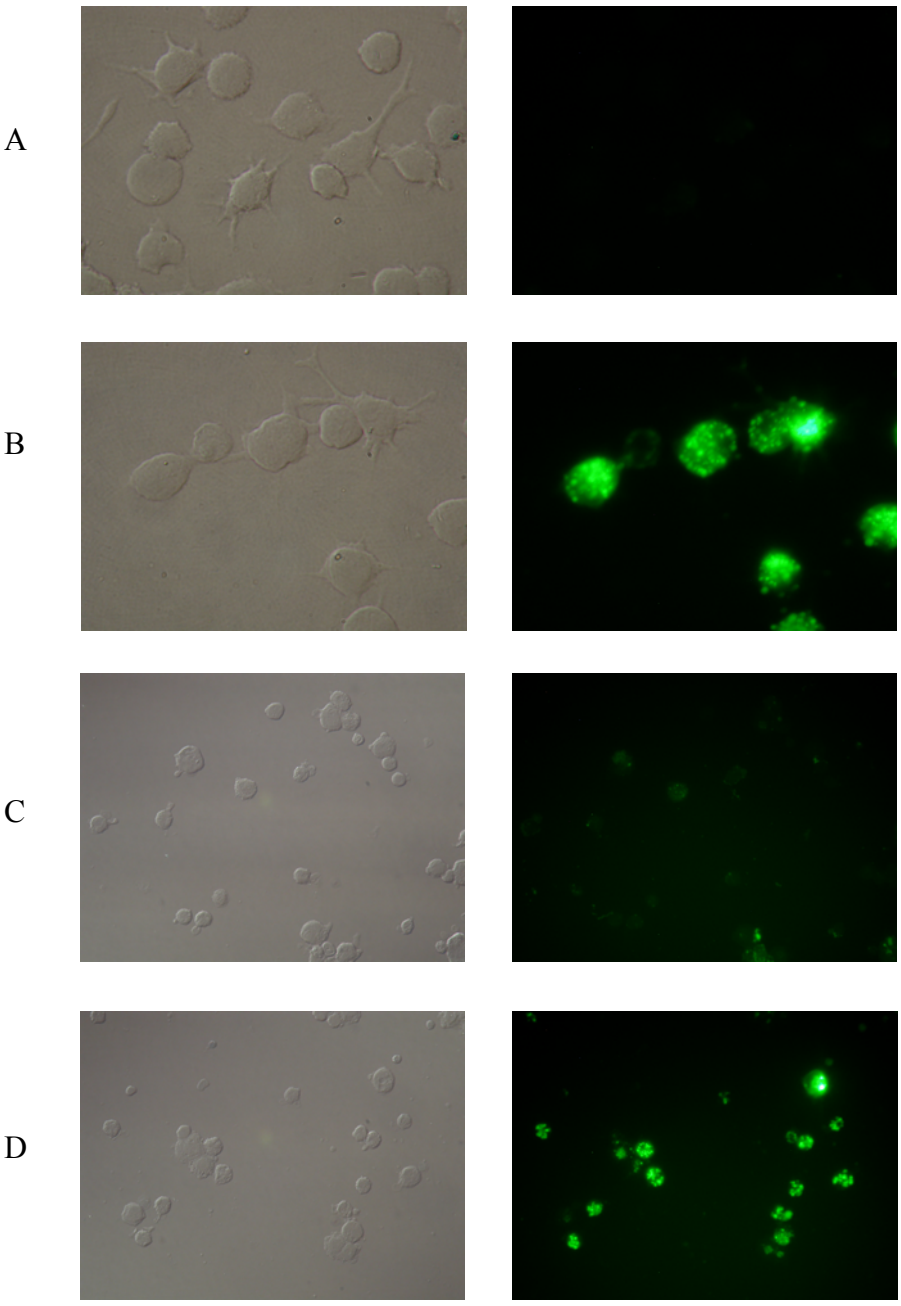


Figure 11. Activated dendritic cells can internalize LPD but not free DNA

DC2.4 (A,B) or primary BMDC dendritic cells (C,D) were activated overnight with LPS then treated for 1 hour with fluorescein-labeled DNA only (A,C) or LPD prepared with fluorescein-labeled DNA (B,D). Cells were washed and viewed at 40X magnification with a wide-field fluorescent microscope.

2.3.2 Intracellular trafficking of LPD

Confocal microscopy experiments using fluorescent LPD nanoparticles for internalization by resting or activated DC2.4 cells indicated that both types of cells can internalize LPD, but that activated cells do so at an increased level (Figure 12). Distinct green structures were visible inside the cells and are indicative of membrane-bound organelles. For that reason and the elimination of membrane-bound LPD as seen by traditional fluorescent microscopy, confocal microscopy was used to determine the subcellular localization of the LPD components.

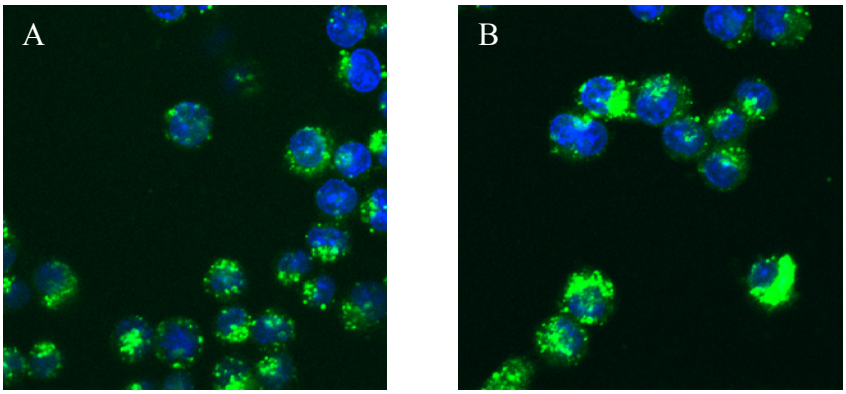


Figure 12. Activated cells have increased vaccine uptake

Resting (A) or LPS-activated (B) DC2.4 cells were incubated for 1 hour with fluorescent LPD before extracellular fluorescence was quenched with trypan blue. Cells were counterstained with DAPI and imaged by confocal microscopy.

LPD nanoparticles were formed using either rhodamine-labeled liposome or Cy3-labeled plasmid DNA and were used to treat immature or mature DC2.4 cells for 1 hour. After treatment, endosomes or lysosomes were labeled with a FITC-tagged secondary antibody to the

organellar-specific primary antibody. Samples were then analyzed for co-localization by confocal microscopy.

Samples incubated with unlabeled LPD particles and visualized with unstained organelles serve as controls verify the experimental conditions are optimal (data not shown). It is clear that the DNA component of the LPD nanoparticle vaccine traffics to both the endosome and lysosome in the activated dendritic cells as shown by co-localization (yellow) (Figure 13). Interestingly, it appears there are very few organized endosomes in the resting dendritic cells. Similar to earlier findings, this data verifies that internalization of LPD particles increased with cellular activation. Additionally, this increase coincided with an increase in the number of acidic vesicles (endosomes and lysosomes) in the cells, which would explain the apparent upregulation of internalization upon activation.

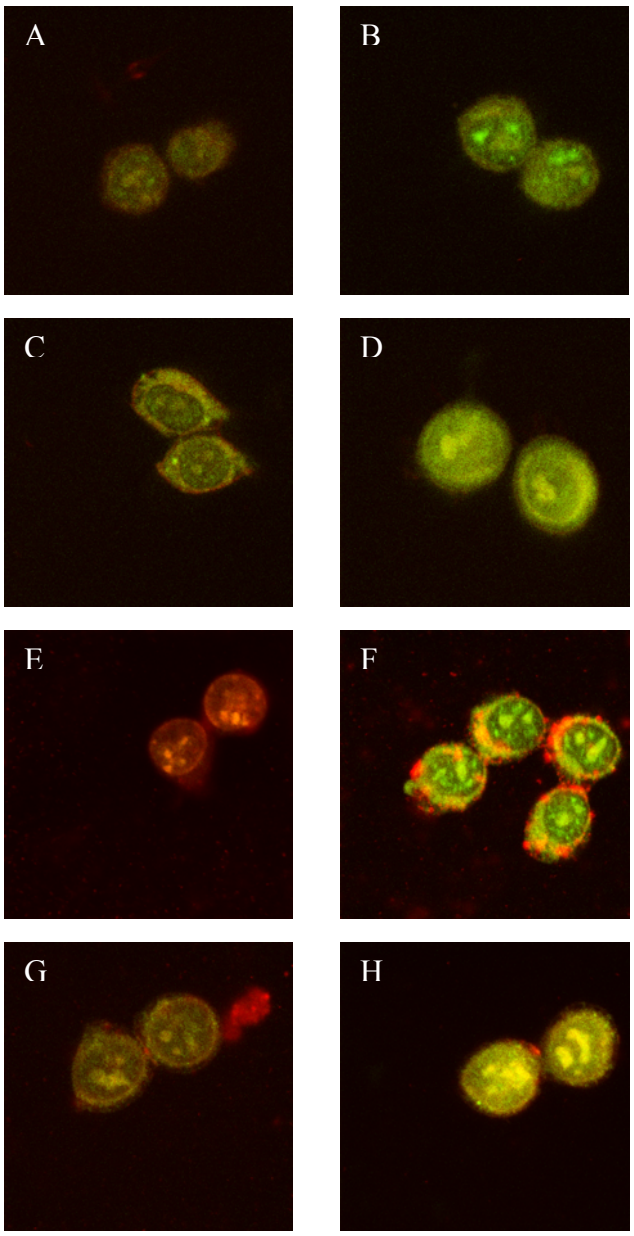


Figure 13. Subcellular localization of LPD nanoparticles in DC2.4 cells

LPD nanoparticles were formed using either Rhodamine liposomes (A-D) or Cy3-labeled DNA (E-H) and were used to treat resting (A,C,E,G) or LPS-activated (B,D,F,H) DC2.4 cells for 1 hour. After treatment, cells were fixed and permeabilized. Endosomes (A,B,E,F) or lysosomes (C,D,G,H) were labeled with a FITC-tagged secondary antibody to the organellar-specific primary antibody. Co-localization was analyzed by confocal microscopy (60X).

2.4 DISCUSSION

Dendritic cells are activated by LPS, LPD nanoparticles, and liposomes. Dendritic cells, whether resting or activated, are capable of internalizing liposomes and LPD nanoparticles but not free DNA.

Contrary to accepted wisdom, activated dendritic cells can internalize LPD nanoparticles, even showing an increase in uptake over resting dendritic cells. The reason behind this observation remains unclear. After using BMDC for microscopy, it seems unlikely that differences between primary dendritic cells and DC2.4 dendritic cells are the cause. It is possible that properties unique to LPD are involved. It is also possible these observations are due to inherent properties of the dendritic cells. The latter possibility initially seemed unlikely, considering the dogma of endocytosis down regulation upon DC activation, but other published work has verified the immediate upregulation of endocytosis upon DC activation [9].

Whether flow cytometry or fluorescent microscopy is used to analyze internalization, a negative response can not be argued. If the results indicate a positive response, or internalization, one may argue that fluorescent particles are merely attaching to the surface of the cells and are not, in fact, internalized. To address this issue and study the internalization in greater detail, confocal microscopy was used to visualize thin sections of the cell interior as opposed to the compound images obtained with a traditional fluorescent microscope.

Similar to earlier findings, internalization of LPD particles increased with cellular activation and coincided with an increase in the number of acidic vesicles (endosomes and lysosomes) in the cells. Additionally, in activated dendritic cells, the DNA portion of LPD

nanoparticles appears to localize in endosomes and lysosomes. This is particularly interesting because the DNA core of the LPD mimics the peptide component of the simple DOTAP/E7 therapeutic vaccine, which is necessary for the induction of an antigen-specific response. Furthermore, the DNA core traffics through the endosomes, presumably where the unmethylated CpG motifs interact with TLR9 to activate the cells in a non-specific manner.

3.0 INTERACTION OF DOTAP/E7 VACCINE WITH DENDRITIC CELLS

3.1 INTRODUCTION

In the absence of immune system activation by TLR stimulation (such as with the unmethylated CpG motifs found in the LPD/E7 vaccine), the vaccine is still capable of eliciting an anti-tumor response *in vivo* [24]. This simple vaccine, consisting of a single cationic lipid and the immunodominant epitope of the human papillomavirus 16 E7 oncogene (DOTAP/E7), generates antigen-specific cytotoxic T lymphocytes *in vivo* toward the regression of established tumors bearing the same E7 peptide [24]. It is hypothesized that dendritic cells internalize the vaccine particles to initiate an anti-E7 adaptive immune response and that DOTAP works both as a protective delivery vehicle for the E7 peptide antigen and as a vaccine adjuvant.

It is important and necessary to discover the ways in which this vaccine interacts with mediators of the adaptive immune system toward development of an anti-tumor response. The goal of these studies is to elucidate the details of the interaction between dendritic cells and the DOTAP/E7 vaccine toward a more comprehensive explanation of the mechanism of vaccine action. To that end, it is imperative to study the internalization and trafficking of the vaccine by dendritic cells.

3.2 MATERIALS AND METHODS

3.2.1 Reagents

1,2-Dioleoyl-3-Trimethylammonium-Propane (DOTAP) and 1-oleoyl-2-[6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]hexanoyl]-3-trimethylammonium propane (18:1-06:0 NBD DOTAP) were purchased from Avanti Polar Lipids (Figure 1). Lipopolysaccharide and the endocytosis inhibitors (Amiloride, Azithromycin, Methyl-β-cyclodextrin, Phenylarsine oxide, Phorbol-12-myristate-13-acetate, Rottlerin, Sucrose, Staurosporine and Tannic acid were purchased from Sigma Chemical Company. PD-98059 and Wortmannin were purchased from Calbiochem. RPMI-1640, non-essential amino acids, antibiotic/antimycotic and PBS were purchased from Gibco Chemical Company and GM-CSF and IL-4 mouse cytokines were purchased from R&D Systems. All antibodies were purchased from Becton Dickinson unless otherwise specified.

3.2.2 Cell culture

Primary dendritic cells were differentiated from murine bone marrow precursors in RPMI-1640 supplemented with 10% FBS, NEAA, antibiotic, antimycotic, GM-CSF and IL-4 for 6 days at 37°C and 5% CO_2 in a protocol adapted by the author from Inaba, *et al* [27]. CD8+ T lymphocytes were purified by depletion (CD8+ T cell isolation kit, Miltenyi Biotec) from freshly prepared naïve splenocytes and maintained in RPMI-1640 growth medium supplemented with

10% FBS, antibiotic, antimycotic, non-essential amino acids and β-mercaptoethanol. Cytokines IL-2 and IL-7 were added where indicated.

3.2.3 Animals

Female C57BL/6 mice at 5-6 weeks of age were purchased from Charles River and used in accordance with Institutional animal care guidelines.

3.2.4 Preparation of DOTAP/E7 vaccine

DOTAP (1,2-dioleyltrimethylammonium propane) liposomes are prepared by thin film hydration followed by sonication and extrusion through a 0.1μm membrane. For fluorescent liposomes, NBD-DOTAP was mixed with DOTAP in chloroform at a 1/100 molar ratio prior to solvent evaporation. To prepare DOTAP/E7 particles, the lipid was hydrated in a peptide solution and unencapsulated E7 was not removed after extrusion. To prepare fluorescent DOTAP/E7 vaccine particles, DOTAP and/or NBD-DOTAP was hydrated with E7 and/or rhodamine-E7 peptide solution, which was prepared at 5 molar percent.

3.2.5 Flow cytometry

Two million cells per sample were washed twice with BD Stain Buffer and resuspended in 200 μl Stain Buffer with 1 μl each fluorophore-conjugated primary antibody, as indicated. Cells were incubated in the dark at 4C for 30 minutes and washed 2 additional times with Stain Buffer. If propidium iodide was used, 2 μl was added to the sample immediately prior to analysis by flow cytometry on a Becton Dickinson FACS Canto. For analysis of NBD-DOTAP liposome

uptake, two million cells per sample were washed twice with PBS to remove serum and resuspended in 250 µl PBS with inhibitor. After incubation at room temperature (or 4C) for 15 min, 250µl NBD-DOTAP liposomes in PBS were added (37.5 µM final concentration of lipid) and incubated at 37C (or 4C) for 1 hour. After incubation, 10% (v/v) Trypan Blue was added and samples were immediately analyzed for NBD fluorescence.

3.2.6 Intracellular cytokine staining

To determine cytokine levels, intracellular flow cytometry was performed. Cells were incubated in the presence of GolgiStop and specific peptide (E7) for 6 hours before fixation. Cells were permeabilized and stained with H-2D[b] DimerX reagent loaded with specific (E7) or irrelevant (NP) peptide and antibodies against CD8 and IFN-γ.

3.2.7 Effector generation

CD8+ T lymphocytes purified from spleens of naïve mice were incubated in the presence of cytokines (IL-2 and IL-7) and treated, fixed dendritic cells. The dendritic cells were treated with 50/20µM DOTAP/E7 vaccine or individual vaccine components at concentrations equivalent to incubation with vaccine for 2 days before fixation with 0.005% glutaraldehyde. Immediately before adding to T cell cultures, dendritic cells were washed three times to remove all traces of fixative. After 2 consecutive 4 day stimulations of the T cells, all groups were re-stimulated with E7 peptide for 2 days. On day 10, assays were performed to test the specificity and functionality of these cells.

3.2.8 CTL assay

Mice were sacrificed and splenocytes were harvested and dissociated. After lysis of red blood cells, total splenocytes (responder cells) were stimulated for 5 days with E7 peptide (5μg/mL) in the presence of 40U/mL recombinant IL-2 (R&D systems, Minneapolis, MN) in complete RPMI-1640 medium. After *in vitro* CTL expansion, responder cells were used as CTL effectors. TC-1 cells were used as target cells in this assay. To discriminate between effectors and targets, TC-1 cells were labeled with PKH-67 (Sigma, St. Louis, MO), a green fluorescent probe, which incorporates into the plasma membrane. TC-1 cells were washed 3 times with RPMI 1640 complete media. To assay the specific killing, effectors and targets were plated into 96-well plates at various effector to target (E:T) ratios and the lysis reaction was carried out for 4 h at 37°C. Cells were harvested and stained with propidium iodide (PI) for analysis on a BD Canto digital flow cytometer (San Diego, CA). Percentage of E7-specific lysis is determined by the percentage of PI-positive cells within the PKH-67-positive region.

3.2.9 Tumor Infiltrating Lymphocytes

Mice were inoculated with $1x10^5$ TC-1 cells subcutaneously 6 days prior to experimentation. Naïve and DOTAP/E7 treated CD8+ T lymphocytes were fluorescently labeled with AF647SE and AF488SE, respectively. Lymphocytes were mixed at a 1:1 ratio and two million total labeled lymphocytes were injected intravenously to tumor-bearing mice. Tumors were removed 24 hours after injection of fluorescent labeled cells. Frozen sections (5μm) were prepared using a cryostat and mounted in media containing DAPI.

3.3 RESULTS

3.3.1 DOTAP/E7 vaccine activates dendritic cells

To validate the hypothesis that dendritic cells generate an anti-E7 immune response when stimulated with the DOTAP/E7 vaccine, the vaccine must first be examined for its potential to activate these cells. Without such activation, delivery of peptide by DOTAP would lead to tolerant dendritic cells that are specific for our peptide rather than 'licensed' dendritic cells capable of generating effector T cells.

To that end, dendritic cells were incubated in the presence of increasing concentrations of vaccine components (DOTAP or E7) or intact vaccine (DOTAP/E7) for a maximum of 48 hours. At the specified time points, dendritic cells were fixed and analyzed for cell surface marker expression by flow cytometry. The surface markers examined were the activation marker CD86 and the MHCI (H2D[b]) and MHCII (IA[b]) molecules necessary for peptide presentation.

As shown in Figure 14, the DOTAP/E7 vaccine causes both a time and concentration dependent increase in the surface expression of CD86 and MHCI on dendritic cells. These experiments were repeated several times with similar results and data from a representative experiment is shown. DOTAP, but not free peptide, causes a similar time and concentration dependent increase (data not shown). DOTAP and the DOTAP/E7 vaccine are capable of activating BMDC, efficiently licensing them for further immune potential.

Interestingly, neither the DOTAP/E7 vaccine nor the individual components causes an increase in MHCII (data not shown), an observation that remains unexplained. It is important to

note that dendritic cells upregulated CD86, MHCI and MHCII surface markers when treated with the potent stimulator, LPS, which indicates the cells, reagents and methods had no problems.

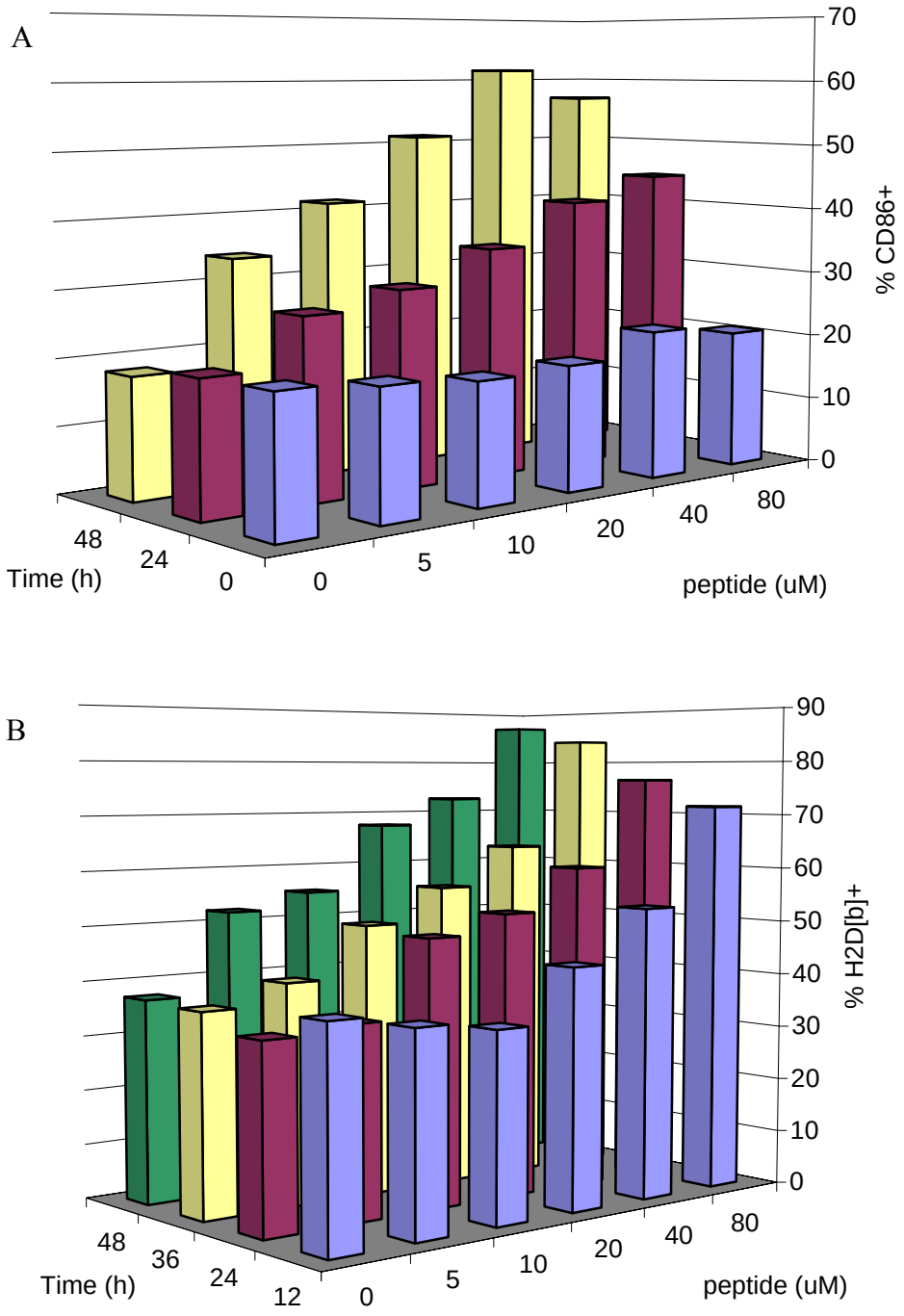


Figure 14. Activation by DOTAP/E7 is both time and concentration dependent

BMDC were incubated with increasing concentrations of DOTAP/E7 vaccine (5/2 molar ratio) for indicated times. Cells were stained for CD86 (A) or MHCI (B) and analyzed by flow cytometry. Representative data is shown.

3.3.2 BMDC internalize DOTAP through multiple endocytic pathways

To determine how the dendritic cells internalize DOTAP for subsequent processing, the cells were incubated with fluorescent DOTAP liposomes (5 molar percent NBD-DOTAP) in the presence of inhibitors for specific endocytosis pathways (Table 2). The goal of this experiment is the interruption and subsequent elucidation of the involved endocytic pathway.

Table 2. Inhibitors of Endocytosis

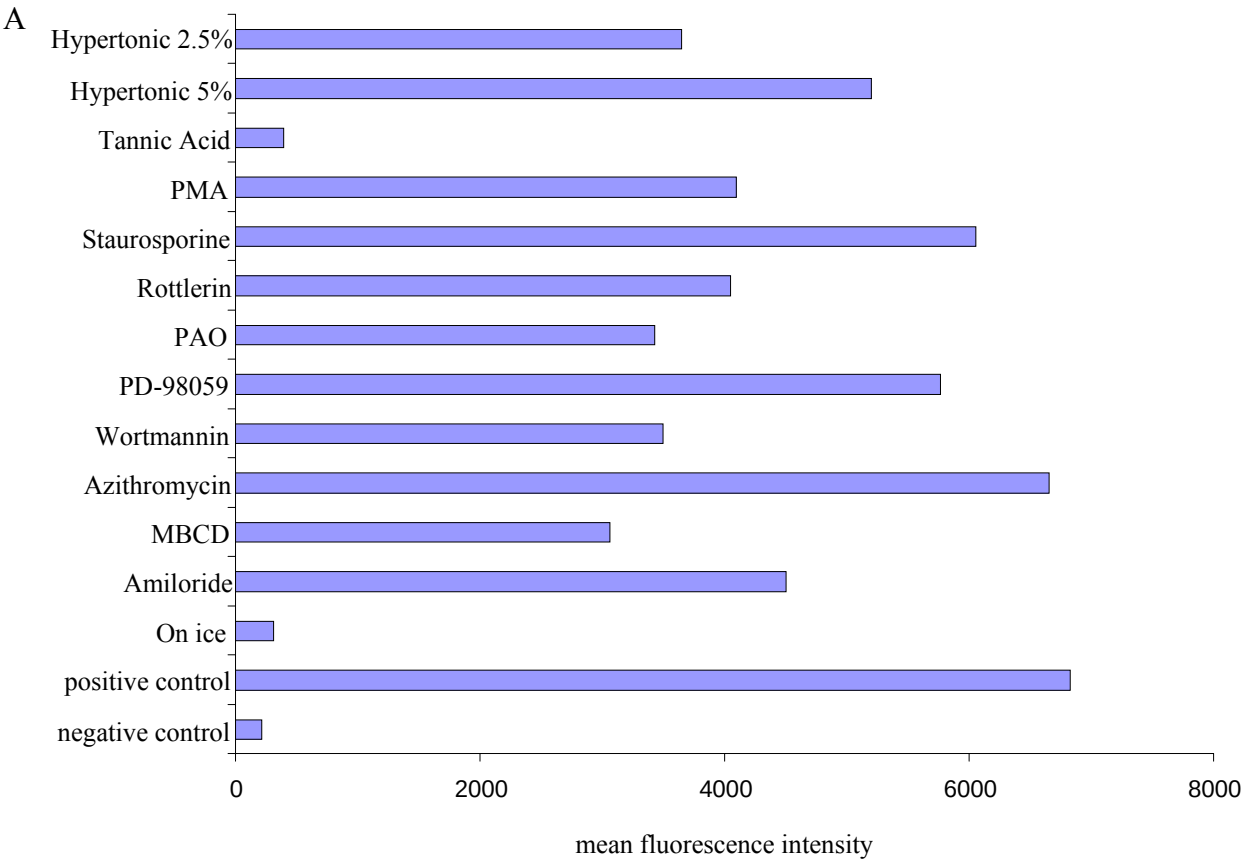
Treatment	Endocytosis pathway inhibited
Amiloride	Fluid Phase Endocytosis (FPE)
Azithromycin	FPE
Hypertonic sucrose	Clathrin-coated vesicle-mediated
Incubation on ice	All endocytosis
Methyl-β-cyclodextrin (MBCD)	Caveolin-dependent and lipid raft-dependent
PD-98059	FPE
Phenyl Arsine Oxide (PAO)	Receptor-mediated
Phorbol-12-myristate-13-acetate (PMA)	Activates PKC (enhances FPE?)
Rottlerin	FPE, but not receptor-mediated
Staurosporine	FPE (inhibits PKC)
Tannic acid	All endocytosis
Wortmannin	Macropinocytosis and Fc-mediated phagocytosis

Specifically, cells were pretreated with inhibitors (ice, 300μM amiloride, 20mM MBCD, 132μM azithromycin, 100nM wortmannin, 20μM PD-98059, 5μM PAO, 40μM rottlerin, 300nM staurosporine, 100nM PMA, 0.02% Tannic Acid and 5 or 2.5% hypertonic sucrose) for 15 minutes. After After incubation at 37C (or 4C) for 1 hour with NBD-DOTAP liposomes, extracellular fluorescence was quenched by the addition of Trypan Blue. BMDC incubated with non-fluorescent DOTAP liposomes and BMDC incubated with NBD-DOTAP at 37C without inhibitors are negative (autofluorescent) and positive control samples, respectively.

Samples were analyzed by flow cytometry and mean fluorescence intensity values of NBD are displayed in Figure 15. As shown, cells incubated without inhibitors (positive control) had high levels of NBD fluorescence. When incubated at 4C, the level of NBD fluorescence is very low and comparable to the cells incubated with non-fluorescent liposomes (negative control). This indicates that Trypan blue does indeed quench extracellular fluorescence. Additionally, this result suggests that internalization of DOTAP by dendritic cells is an active process, which is inhibited at the reduced temperature. Incubation with specific inhibitors showed a partial decrease in fluorescence intensity of the cells, suggesting that each pathway was not the sole method of internalization. Only the tannic acid, which is reported to inhibit all endocytosis, could, in fact, inhibit endocytosis to a level comparable to the reduced temperature treatment.

Dendritic cells treated in the same manner as above were also mounted using GelMount and visualized by confocal microscopy the following day. Representative photographs are also shown in Figure 15. The confocal images confirm the findings of the flow cytometry experiment. Specific inhibitors such as MBCD can only partially reduce uptake whereas treatment with the pan endocytosis inhibitor, tannic acid, can substantially eliminate uptake of DOTAP by the dendritic cells. These experiments were repeated several times with similar results. Results from a representative experiment are shown.

Through these experiments, it was determined that dendritic cells internalize DOTAP through an active process. Additionally, dendritic cells are promiscuous in the use of endocytosis pathways to internalize DOTAP such that an individual endocytic pathway cannot be inhibited in order to abrogate uptake of DOTAP by dendritic cells.



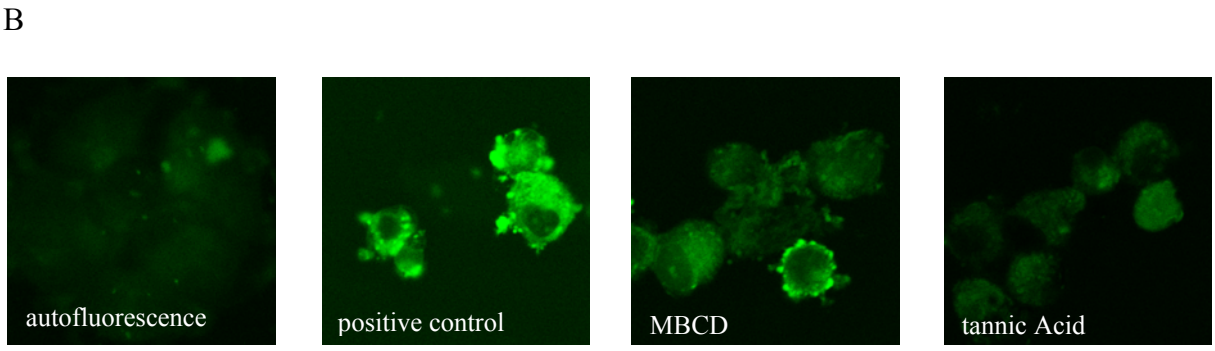


Figure 15. Dendritic cells have multiple means of internalization

Cells were pretreated with inhibitors before incubation with NBD-DOTAP liposomes at 37C. Extracellular fluorescence was quenched after incubation and samples were analyzed by flow cytometry (A) and confocal microscopy (B). BMDC incubated with non-fluorescent DOTAP liposomes and BMDC incubated with NBD-DOTAP at 37C without inhibitors are autofluorescent (negative) and positive control samples, respectively.

3.3.3 Vaccine components traffic to lysosomes

It is important to understand how the vaccine is trafficked through the cell once it's internalized. The trafficking must lead to peptide presentation on MHCI if effectors are to be generated during an adaptive immune response. To this end, DOTAP/E7 vaccine (50/20μM) was prepared with fluorescently labeled DOTAP (NBD), peptide (rhodamine) or both. BMDC were incubated with fluorescent vaccine and co-localization studies were conducted (Figure 16). If fixed cells were analyzed, the samples were first counterstained with DAPI.

Live (E) or fixed (A) BMDC were incubated with the double fluorescent NBD-DOTAP/Rhodamine-E7 vaccine. As shown in Figure 16, the live cells show the intact vaccine is localized in the same vesicle; whereas the fixed cell images shows the vaccine has separated into distinct compartments. The lipid component appears mostly cytosolic while the peptide component is localized in punctuate, perinuclear structures, which, presumably, are lysosomes.

Dendritic cells were then treated with LysoTracker Red (B-D), which accumulates in acidic cellular compartments and is used to label lysosomes [28, 29]. Upon incubation with fluorescent green lipid (NBD-DOTAP/E7), it is possible to verify the presence of DOTAP in lysosomes as evident by the co-localization (yellow).

Dendritic cells were then incubated with only red fluorescent peptide (DOTAP/rhodamine-E7) before fixation and visualization (F). Again, it is possible to see the peptide in defined perinuclear structures.

LysoTracker Green and red fluorescent peptide (DOTAP/rhodamine-E7) were used to determine if peptide trafficked to lysosomes, the presumed perinuclear structures. As shown in Figure 16G live cell image, the peptide does, indeed, traffic to the lysosomes.

Furthermore, the dendritic cells were incubated with red fluorescent peptide (DOTAP/rhodamine-E7) and phalloidin-AF488, which stains actin green. Co-localization in this example is difficult to see, indicating that if the peptide had been cytosolic, it was not residing in actin-rich regions.

These results indicate the DOTAP/E7 vaccine is mostly trafficked through the vacuolar pathway, presumably where the peptide is loaded onto surface-recycled MHCI for surface presentation. MHCI has a conserved cytoplasmic tyrosine-based endocytic sorting motif, YXXA [30]. This pathway plays a central role in cross-presentation by dendritic cells, which is crucial in the development of an antigen-specific CTL response to viral and tumor antigens. The traditional cross-presentation pathway wherein cytosolic proteins that were released from the endosomes are degraded by the proteasome and enter the endoplasmic reticulum via the TAP protein must still be reconciled. Indeed, at very early time points, there is evidence of cytosolic peptide (Figure 17) as visualized by a diffuse distribution. It is unclear whether all peptide is cytosolic and then routed to the vacuolar pathway or these are 2 distinct pathways for presentation via MHCI.

Evident in Figures 15 and 16, DOTAP distribution has been shown to have a diffuse pattern: evidence that DOTAP is also cytosolic at some point during the internalization process. Again, it remains unclear whether these two pathways are distinct. Both components of the DOTAP/E7 vaccine traffic through the vacuolar pathway and at some point reside in the cytoplasmic compartment.

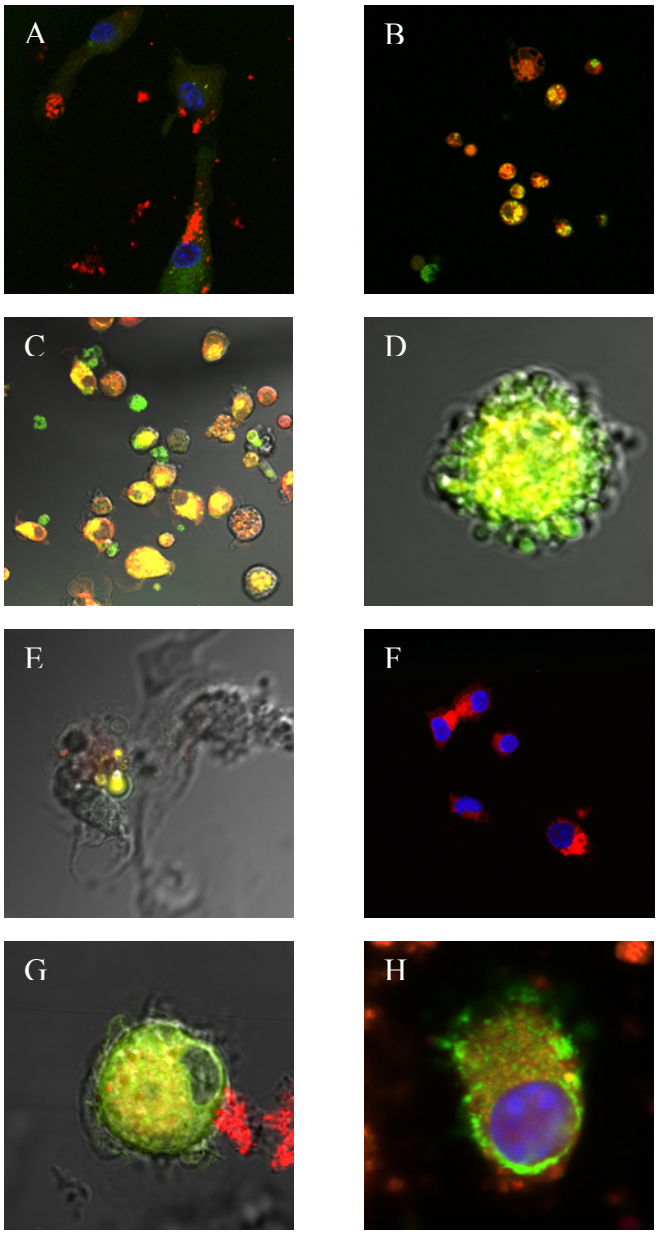


Figure 16. Vaccine components traffic to lysosomes

Live (B-E, G) or fixed (A,F,H) BMDC were incubated with NBD-DOTAP/Rhodamine-E7 (A+E), NBD-DOTAP/E7 (B-D) or DOTAP/Rhodamine-E7 (F-H) vaccine for visualization by confocal microscopy. Fixed cells were counterstained with DAPI. BMDC were incubated with LysoTracker Red (B-D), LysoTracker Green (G) or Phalloidin-488 (H) for co-localization studies.

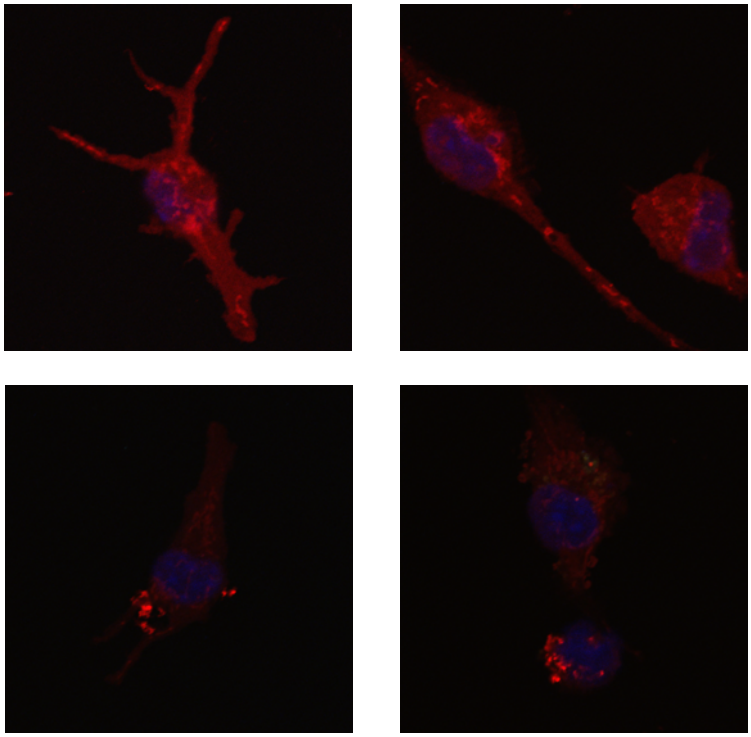


Figure 17. At very early time points, peptide is cytosolic

BMDC were incubated with DOTAP/Rhodamine-E7 for 1 minute (top row) or 10 minutes (bottom row) before extracellular fluorescence was quenched. Samples were immediately fixed, then counterstained with DAPI and visualized by confocal microscopy. Two unique fields are shown for each time point.

3.3.4 Generation of antigen-specific T lymphocyte effectors

BMDC are capable of internalizing vaccine-associated peptide and traffic that peptide through the vacuolar pathway for cross-presentation. Additionally, these cells up-regulate MHCI on their surface in response to stimulation with DOTAP. The next step was to determine if the dendritic cells present peptide via MHCI on the surface. Attempts to directly examine peptide presentation were unsuccessful largely because of technical problems. Therefore, peptide presentation was examined indirectly by way of examination of resultant effectors.

3.3.4.1 *In vitro* effector generation

The DOTAP/E7 vaccine was incubated with dendritic cells for 2 days at which time the cells were fixed and added to cultures of naïve CD8+ T cells. If it is possible to recapitulate the effector generation process in vitro, the effector T cells generated from incubation with the autologous dendritic cells would proliferate and be both antigen specific and functional.

Effector proliferation

After 2 consecutive 4 day stimulations with treated, fixed dendritic cells, all groups were re-stimulated with E7 peptide for 2 days. On day 10, assays were performed to test the specificity and functionality of these T cells.

First, it was examined whether incubation with treated dendritic cells would cause proliferation and clonal expansion of the T cells. As shown in Figure 19, after 8 days in culture, there are visible clusters of cells that would not dissociate after washing, indicative of clonal expansion caused by the treated, fixed dendritic cells.

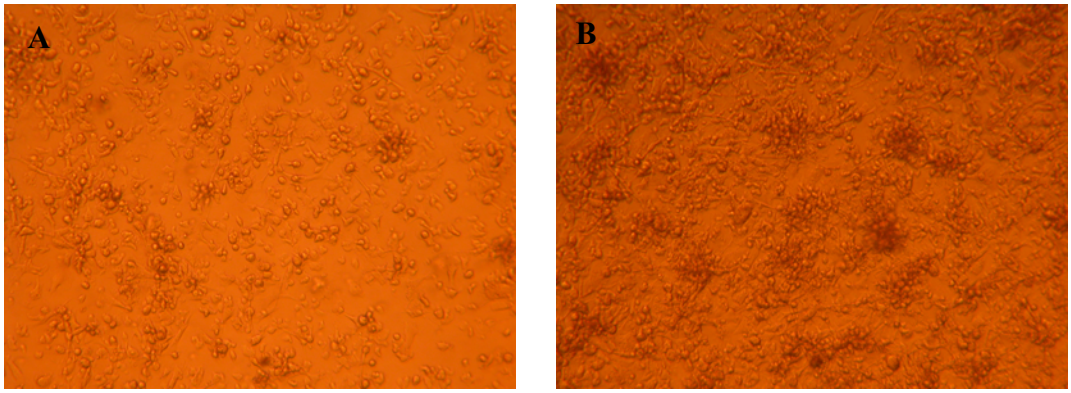


Figure 18. Photographic evidence of effector proliferation

CD8+ T lymphocytes purified from naïve spleens were incubated with cytokines (IL-2 and IL-7) and fixed dendritic cells that had been treated with DOTAP/E7 (B) or E7 only (A) for 2 days. CD8+ cells received treated, fixed dendritic cells twice (days 0 and 4). Pictures of the cultures were taken 8 days after first treatment.

TCR Specificity

Specifically, the cells were tested for TCR specificity by flow cytometry using the DimerX reagent (Becton Dickinson), which is a fusion molecule consisting of an immunoglobulin and 2 MHC molecules (H2D[b] in this case) in a configuration similar to MHC tetramers. The DimerX was loaded with specific (E7) or irrelevant (NP) peptide overnight according to manufacturer's instructions the labeled with secondary antibodies. The cells were stained with each Dimer and analyzed by flow. These results, shown in Figure 20, demonstrate the cells are indeed specific for the E7 peptide. Using the DimerX reagent, cells stain positive for the E7 peptide, regardless of the fluorophore on the secondary antibody, discounting the fluorophore as a possible reason for this positive data.

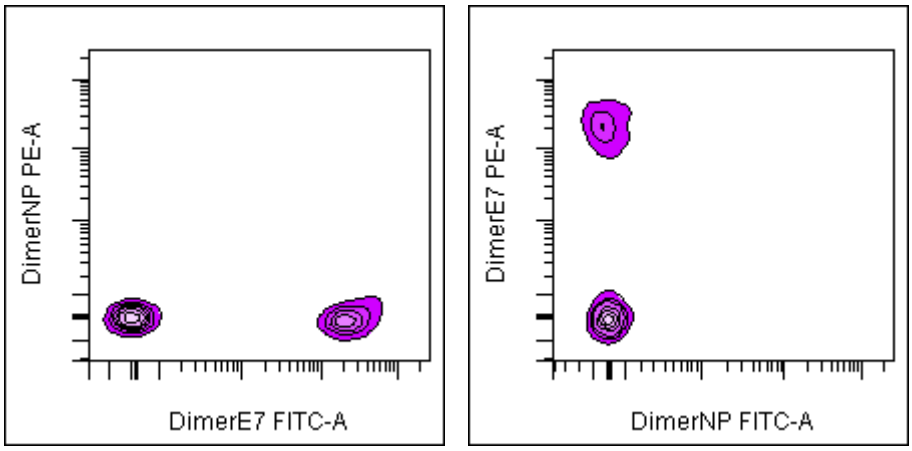


Figure 19. CTL specifically recognize MHC/E7

Cells were stained with CD8 and H-2D[b] DimerX reagent loaded with specific (E7) or irrelevant (NP) peptide and analyzed by flow. CD8+ cells gated.

This experiment was then conducted using only FITC-conjugated secondary antibody for the DimerX reagent. Cells were treated as described above and stained for CD8 (APC) and DimerX (FITC). After the experiment was completed using two separate batches of cells, the data was pooled and analyzed for the percent of cells that were Dimer positive within the CD8+ population. The cells stained with DimerNP and DimerE7 were 0.3% and 43.6% DimerX+/CD8+, respectively, indicating the cells specifically recognize the MHC/peptide complex. A 2-tailed, unequal variance t test conducted on the pooled data showed the DimerNP and DimerE7 groups were significantly different, with a p-value less than $4x10^{-6}$.

Functional specificity

Furthermore, intracellular cytokine analysis was conducted to determine the functionality of the cells as production of IFN-γ correlates with CTL effector functionality [31]. Intracellular cytokine analysis allows for determination of cytokine-producing cells on a single cell level.

After 10 days in culture, CD8+ T lymphocytes incubated with DOTAP/E7 treated dendritic cells (DC-D/E) were incubated 6 hours in the presence of GolgiStop and specific (E7) peptide. Cells were fixed and stained for CD8, Dimer and IFN-γ. CD8+ cells were gated and results were plotted for Dimer and IFN-γ staining. These experiments were repeated at least 3 times and representative dot plots are shown in Figure 21. Effectors stained with DimerNP and DimerE7 were 0.0% and 3.8% IFN-γ+Dimer+/CD8+. These results indicate that the effectors are both functional (produce IFN-γ) and specific.

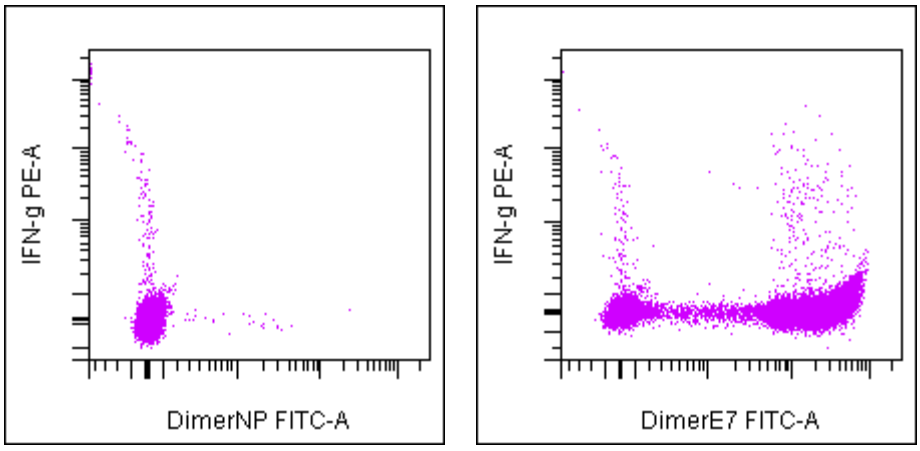


Figure 20. CTL produce IFN-gamma antigen-specifically

Cells were incubated 6 hours in the presence of specific (E7) peptide and GolgiStop. IFN-gamma production was detected by intracellular cytokine staining. Cells were also stained with CD8 and H-2D[b] DimerX reagent loaded with specific (E7) or irrelevant (NP) peptide and analyzed by flow. CD8+ cells gated.

In vivo functionality

As discussed above, naïve CD8+ T lymphocytes that are cultured with DOTAP/E7-treated dendritic cells (DC-D/E) possess a T cell receptor specific for the H2D[b]/E7 MHCI/peptide complex. Furthermore, the CD8+ T cells cultured with DC-D/E were shown to be functional (produce IFN-γ), which correlates with tumor infiltration, an essential component to effector function (10.1038/msb.2009.15).

Results so far support the hypothesis that dendritic cells incubated with DOTAP/E7 mediate an anti-E7 adaptive immune response. However, if the T cells cannot home to the tumor, the location of their effector function, these results are meaningless. To address this issue, the cultured T cells were introduced to tumor-bearing mice and the tumors visualized the next day for the presence of tumor infiltrating lymphocytes. Specifically, at the conclusion of culture (day 10), CD8+ T cells incubated with DC-D/E were labeled with AF488SE (green) and mixed with freshly prepared naïve CD8+ T cells that were labeled with AF647SE (red). The

cells were mixed at a 1:1 ratio and injected intravenously into TC-1 tumor-bearing mice. Twenty four hours later, tumors were harvested. A cryostat was used to prepare frozen sections (5μm) that were mounted with a medium containing DAPI for visualization by confocal microscopy. Images were acquired from different slices and the 3 color overlays are shown in Figure 22. Tumor nuclei were stained with DAPI and appear blue. Naïve and DC-D/E treated CD8+ T cells appear red and green, respectively, in these overlays. Note the DAPI-stained tumor nuclei and green fluorescent, E7-specific CD8+ infiltrating lymphocytes and absence of naïve (red) CD8+ lymphocytes in the tumor sections.

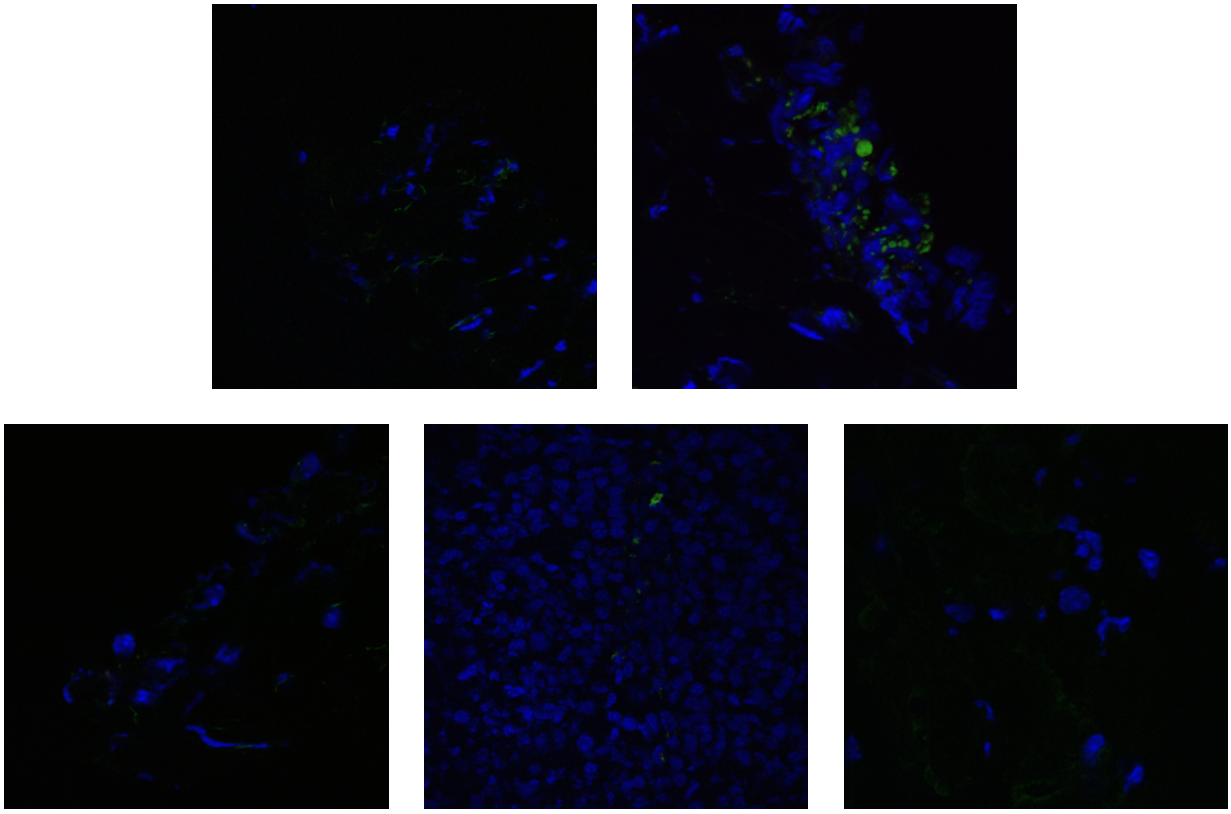


Figure 21. Tumor infiltrating lymphocytes

Confocal microscopy images of tumor sections removed from mice 24 hours after naïve (red) or DC-D/E treated (green) fluorescent-labeled CD8+ T lymphocytes were mixed and injected intravenously. Five unique fields are presented.

3.3.4.2 *Ex vivo* effector generation

To further confirm dendritic cells are capable of producing CTL effectors through peptide presentation, dendritic cells were incubated with DOTAP/E7 vaccine *ex vivo* to confer immunity to mice *in vivo*, as verified by specific CTL killing of tumor target cells. BMDC were incubated *ex vivo* with DOTAP (70μg), E7 peptide (10μg), DOTAP/E7 or left untreated for 1 hour at 37C. The cells were washed three times and 10^6 cells were injected intravenously into each naïve mouse. The mice were inoculated with TC-1 tumor (10^5 cells subcutaneously per mouse) on day 7 and spleens harvested for responder cells on day 17. After a 5 day *in vitro* re-stimulation with E7 peptide, the effector cells were used in a CTL assay with TC-1 cells as the target cells (Figure 18) as described in the methods section. E7-specific lysis was calculated as the percentage of PI-positive cells within the PKH-67-positive region. As shown, only the mice injected with DOTAP/E7-treated dendritic cells were able to generate CTL specific for the E7 positive TC-1 tumor cell line. This confirms earlier results, whereby dendritic cell treatment with DOTAP/E7 vaccine initiates an antigen specific adaptive immune response.

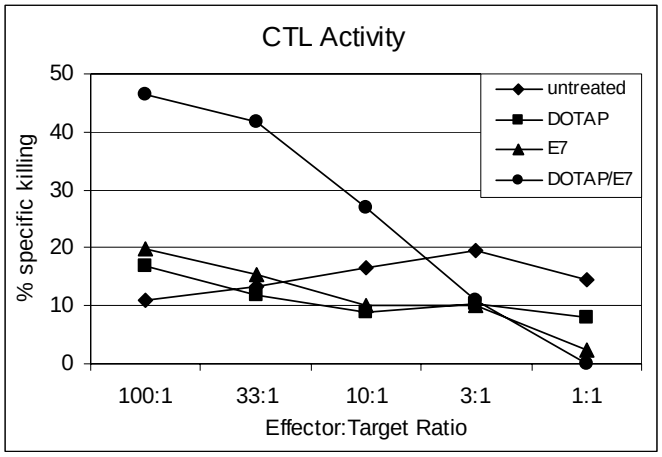


Figure 22. Cytotoxic T Lymphocyte assay from *ex vivo* dendritic cells

BMDC were treated for 1 hour at 37C with indicated formulations. Cells were washed and injected to naïve mice via tail vein. Tumors were inoculated 7 days after dendritic cells were injected. Spleens were harvested 10 days after tumor inoculation for CTL assay with TC-1 targets.

3.4 DISCUSSION

BMDC activation by the DOTAP/E7 vaccine (and DOTAP alone but not free peptide) is both time and concentration dependent as shown by an upregulation of CD86 and MHCI surface markers. Clearly, there is time dependence because of the time required for upregulation of surface markers. Interestingly, surface MHCI but not MHCII is upregulated in this manner. The mechanism behind this observation remains unclear and warrants further study. Additionally, the fact that the DOTAP/E7 vaccine doesn't upregulate surface MHCII has implications for future applications of DOTAP for modulation of the immune response.

Bone marrow-derived dendritic cells use many endocytosis pathways to internalize the DOTAP/E7 vaccine via an active internalization process. The DOTAP/E7 vaccine delivers the peptide cargo to dendritic cells, which in turn, traffic the vaccine components through the vacuolar pathway for cross presentation of the peptide.

These DOTAP/E7 treated dendritic cells are sufficient to generate specific and functional effectors both *in vitro* (when incubated with naïve CD8+ T cells) and *in vivo*. Incubating naive CD8+ T lymphocytes with DOTAP/E7-treated dendritic cells cause clonal expansion of the effectors. Additionally, these effectors are specific (have T cell receptors specific for H2D[b]/E7), functional (produce IFN-γ) and capable of homing to the tumor environment *in vivo*. Furthermore, *ex vivo*-treated dendritic cells re-administered to autologous mice are capable of generating functional, E7 specific CTL effectors *in vivo*.

The DOTAP/E7 vaccine functions to both deliver antigenic peptide as well as 'license' the dendritic cells for the development of antigen specific effectors.

4.0 INTERACTION OF DOTAP/E7 VACCINE WITH CD8+ T LYMPHOCYTES

4.1 INTRODUCTION

Previous unpublished observations by this group present the possibility that in a human model in vitro, the DOTAP/E7 vaccine causes T cell proliferation and generates CTL in the absence of dendritic cells. These were exciting findings, but without appropriate histocompatible controls, it was impossible to determine if this was experimental artifact or actual antigen-specific responses. Regardless, these findings were cause for further study and the reason for the subsequent experiments. If the generation of effectors *in vitro* using only a simple lipid/peptide system is possible, then the potential applications for this technology are astounding.

Foremost, it was important to have purified cells readily accessible. If CD8+ T lymphocytes are allowed to be cultured in the presence of other cell types, many more variables are introduced that will cloud the data. Additionally, when it is observed from a potential clinical standpoint, the purest sample would be the best. Less variation among patients should occur if a purified starting point is utilized.

Particular issues that required attention include the activation potential, the possible mitogenic properties and the signaling initiation caused by DOTAP/E7. Additionally, it was important to re-evaluate earlier ideas that DOTAP/E7 could indeed generate antigen-specific effectors in vitro in the absence of antigen presenting cells in the context of appropriate controls.

4.2 MATERIALS AND METHODS

4.2.1 Reagents

1,2-Dioleoyl-3-Trimethylammonium-Propane (DOTAP) and 1-oleoyl-2-[6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]hexanoyl]-3-trimethylammonium propane (18:1-06:0 NBD DOTAP) were purchased from Avanti Polar Lipids (Figure 1). Phytohemagglutinin (PHA) and Phorbol-12-myristate-13-acetate (PMA) were purchased from Sigma Chemical Company. RPMI-1640, non-essential amino acids, antibiotic/antimycotic, β-mercaptoethanol and PBS were purchased from Gibco Chemical Company. IL-2 and IL-7 cytokines were purchased from R&D Systems. The mouse CD8+ T cell isolation kit was purchased from Miltenyi Biotec. All other antibodies were purchased from Becton Dickinson unless otherwise specified.

4.2.2 Cell culture

CD8+ T lymphocytes were purified by depletion (CD8+ T cell isolation kit, Miltenyi Biotec) from freshly prepared naïve splenocytes and maintained in RPMI-1640 growth medium supplemented with 10% FBS, antibiotic, antimycotic, non-essential amino acids and β-mercaptoethanol. Cytokines IL-2 and IL-7 were added where indicated.

4.2.3 Animals

Female C57BL/6 mice at 5-6 weeks of age were purchased from Charles River and used in accordance with Institutional animal care guidelines.

4.2.4 Preparation of DOTAP/E7 vaccine

DOTAP (1,2-dioleyltrimethylammonium propane) liposomes are prepared by thin film hydration followed by sonication and extrusion through a 0.1μm membrane. For fluorescent liposomes, NBD-DOTAP was mixed with DOTAP in chloroform at a 1/100 molar ratio prior to solvent evaporation. To prepare DOTAP/E7 particles, the lipid was hydrated in a peptide solution and unencapsulated E7 was not removed after extrusion. To prepare fluorescent DOTAP/E7 vaccine particles, DOTAP and/or NBD-DOTAP was hydrated with E7 and/or Rhodamine-E7 peptide solution, which was prepared at 5 molar percent.

4.2.5 Flow cytometry

Two million cells per sample were washed twice with BD Stain Buffer and resuspended in 200 μl Stain Buffer with 1 μl each fluorophore-conjugated primary antibody, as indicated. Cells were incubated in the dark at 4C for 30 minutes and washed 2 additional times with Stain Buffer. If Propidium Iodide was used, 2 μl was added to the sample immediately prior to analysis by flow cytometry on a Becton Dickinson FACS Canto.

4.2.6 Peptide uptake by CD8+ cells

Freshly purified CD8+ T lymphocytes were incubated with DOTAP/rhodamine-E7 vaccine (50/20uM) or free rhodamine-E7 peptide (20uM) or left untreated for 30 minutes at 37C. Trypan blue (10% v/v) was added to quench extracellular fluorescence and cells were washed three times with PBS. After analysis by flow cytometry, cell aliquots were resuspended in mounting media with DAPI and visualized by confocal microscopy.

4.2.7 CTL generation

CD8+ T lymphocytes purified from spleens of naïve mice were incubated in the presence of cytokines (IL-2 and IL-7) and 50/20uM DOTAP/E7 vaccine or individual vaccine components at concentrations equivalent to incubation with vaccine. After 2 consecutive 4 day stimulations, all groups were re-stimulated with E7 peptide for 2 days. On day 10, assays were performed to test the specificity and functionality of these cells.

4.2.8 ELISPOT

Untreated or DOTAP/E7 treated CD8+ T lymphocytes at day 10 of culture were incubated 24 hours in the presence of irrelevant (NP), specific (E7) or no peptide. IFN-γ production was determined by ELISPOT assay using the Becton Dickinson mouse IFN-γ kit according to manufacturer's instructions, counted using the Immunospot reader (Cellular Technologies Ltd) and plotted as IFN-γ producing cells per million.

4.2.9 Tumor Infiltrating Lymphocytes

Mice were inoculated with $1x10^5$ TC-1 cells subcutaneously 6 days prior to experimentation. Naïve and DOTAP/E7 treated CD8+ T lymphocytes were fluorescently labeled with AF647SE and AF488SE, respectively. Lymphocytes were mixed at a 1:1 ratio and two million total labeled lymphocytes were injected intravenously to tumor-bearing mice. Tumors were removed 24 hours after injection of fluorescent labeled cells. Frozen sections (5 um) were prepared using a cryostat and mounted in media containing DAPI.

4.3 RESULTS

Splenocytes were prepared from naïve C57BL/6 mice and processed for lymphocyte purification. Using a magnetic activated cell sorting (MACS) depletion protocol, it is possible to purify CD8+ T lymphocytes that have not been stimulated by antibodies. In this procedure, non-CD8+ T cells are bound by biotin-conjugated specific antibodies. Anti-biotin magnetic beads are then incubated with the cells. A magnetic field is applied and all non-CD8+ T cells remain magnet-bound while the untouched, purified CD8+ T lymphocytes are released.

A multi-color flow cytometry experiment was performed to evaluate results of the purification process each time cells were purified as a quality control measure. The full 5 color profile for CD8+ T lymphocyte purification from splenocytes is shown in Figure 23. Clearly, non T cells were removed from the culture. To examine the makeup of the resultant T cells, plots showing the before and after CD3 and CD8 characteristics are plotted. As shown in Figure 24, the remaining T cells were indeed CD8+. By this method, we are able to purify unadulterated CD8+ T lymphocytes for experimental analysis *in vitro* without the contaminating effects of other cell types.

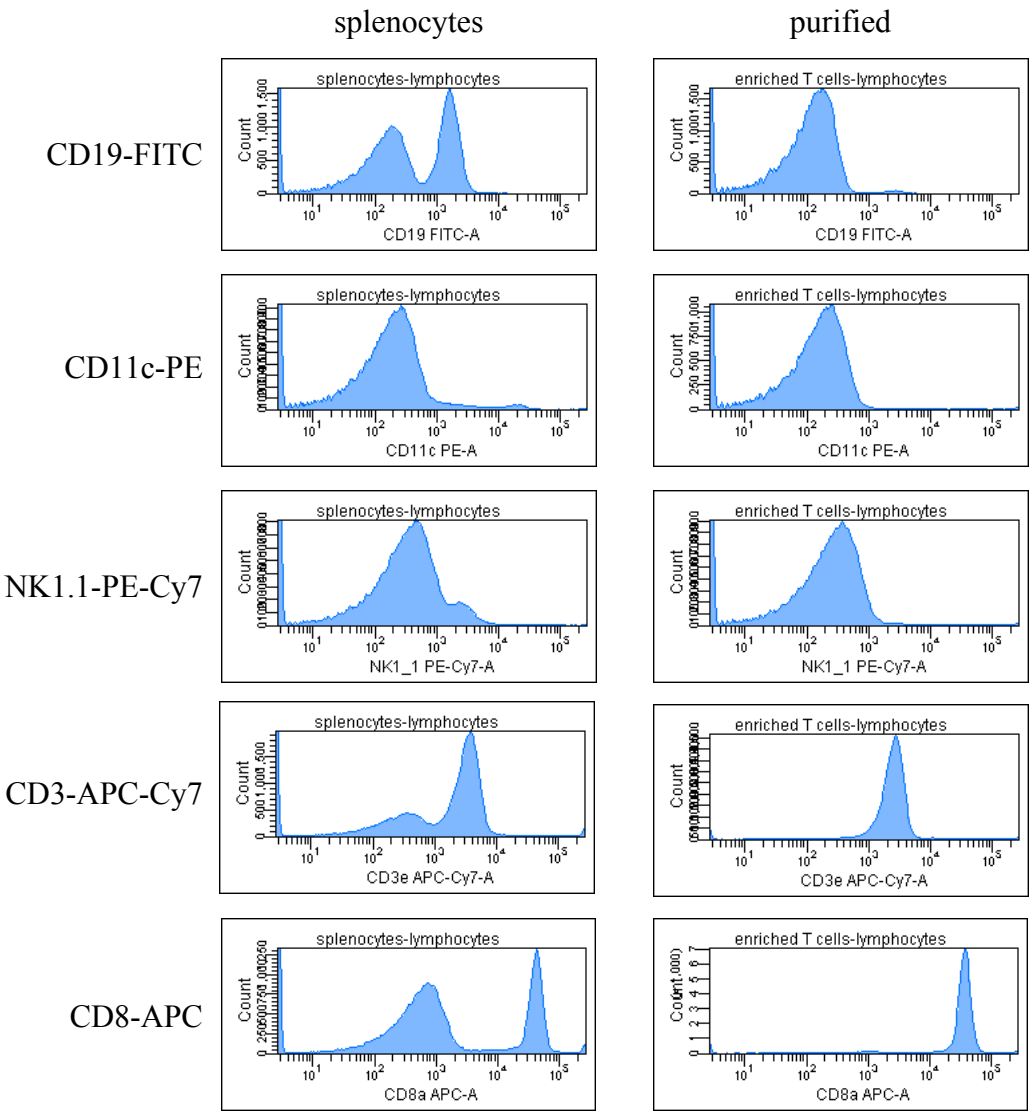


Figure 23. Surface marker profile of CD8+ T cell purification

Five color flow cytometry profile of cells before and after purification of splenocytes for CD8+ T lymphocytes using a MACS depletion protocol.

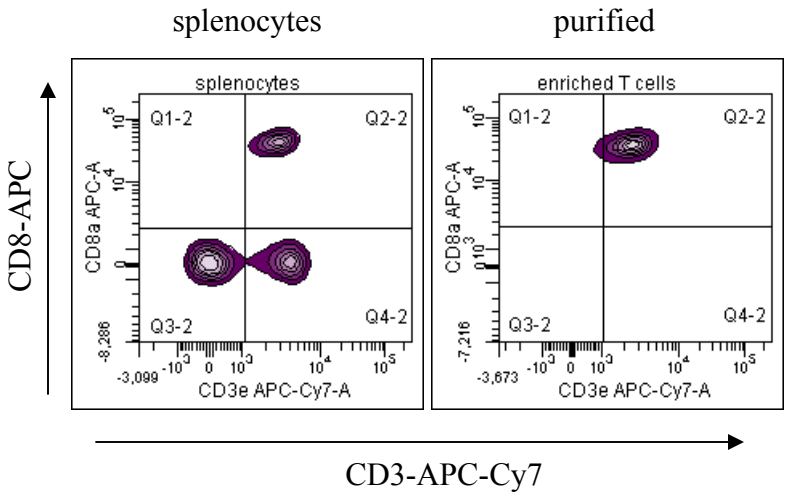


Figure 24. Purification of CD8+ T cells

Contour plot of T cells before and after CD8+ purification.

4.3.1 Activation of cells

CD8+ T cells freshly isolated from naïve spleens were incubated with known (PHA) and prospective (DOTAP) mitogens for 20 hours, stained for the CD69 very early activation marker and analyzed by flow cytometry. As shown in Figure 25, the cells had a concentration dependent increase in surface expression of CD69 when incubated with PHA and DOTAP. Indicating, indeed, the cells were activated by DOTAP.

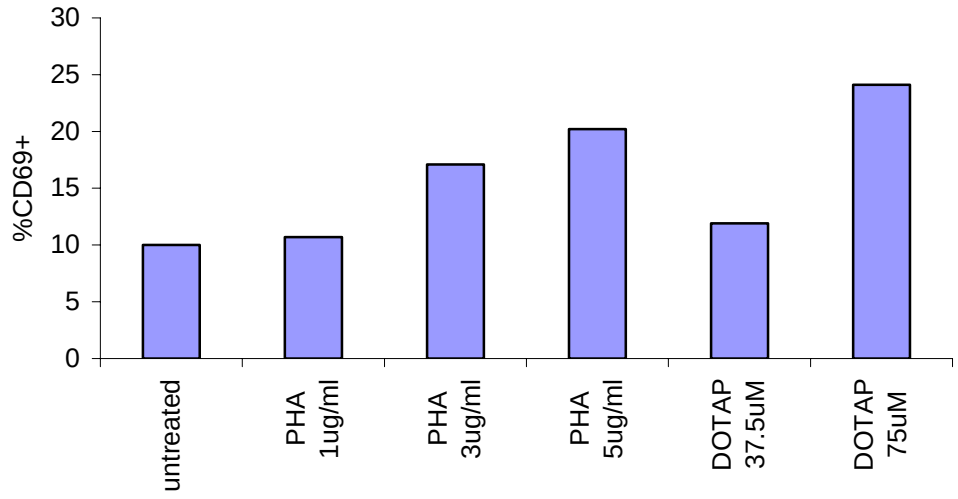


Figure 25. CD8+ T cell activation by mitogens

CD8+ T cells freshly isolated from naïve spleens were incubated in the presence of indicated mitogens for 20 hours before analysis of CD69 expression by flow cytometry.

4.3.2 Internalization of vaccine

If the DOTAP is capable of activating the T cells, it was questioned whether the lipid can also deliver the peptide cargo directly to the intracellular environment. If so, this finding could lead to additional applications for the DOTAP lipid in the context of disease correction.

To address this issue, freshly purified CD8+ T lymphocytes were incubated with DOTAP/rhodamine-E7 vaccine or rhodamine-E7 peptide for 30 minutes at 37C. Extracellular fluorescence was quenched with Trypan blue and cells analyzed by flow cytometry. As shown in Figure 26, the fluorescent peptide was internalized by the cells only when co-formulated with DOTAP. The gate for rhodamine uptake was set for untreated cells at 25.8% positive. Cells incubated with free peptide and DOTAP/E7 were 29.8 and 73.9 percent positive for peptide uptake, respectively, indicating a definite internalization of peptide in the context of vaccine. It is worth noting that only a subset of the T cells internalized the peptide. Aliquots of these samples were then counterstained with DAPI, mounted and visualized by confocal microscopy. Confocal microscopy data (Figure 27) confirms the results of the flow cytometry, that cells internalize peptide in the context of the vaccine but not peptide alone.

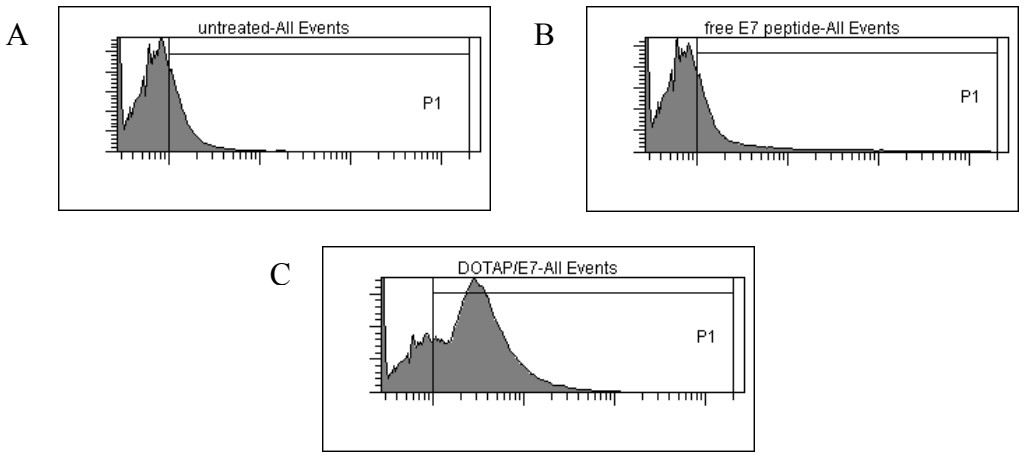


Figure 26. Internalization of E7 peptide by flow cytometry

Freshly purified CD8+ T lymphocytes were incubated with DOTAP/rhodamine-E7 vaccine (C), free rhodamine-E7 peptide (B) or left untreated (A) for 30 minutes at 37C. Extracellular fluorescence was quenched with Trypan blue and cells analyzed by flow cytometry for rhodamine fluorescence.

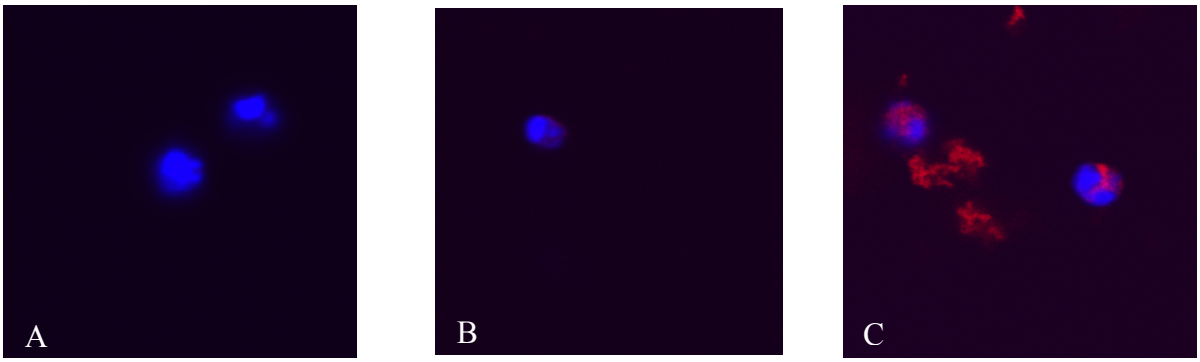


Figure 27. Internalization of E7 peptide by confocal microscopy

Freshly purified CD8+ T lymphocytes were incubated with DOTAP/rhodamine-E7 vaccine (C), free rhodamine-E7 peptide (B) or left untreated (A) for 30 minutes at 37C before extracellular fluorescence was quenched with Trypan blue. Cells were mounted with DAPI and visualized by confocal microscopy.

4.3.3 Mitogenic properties of vaccine

Proliferation and clonal expansion of T cells with a TCR specific for a particular MHC/peptide complex is crucial to the development of an adaptive immune response against that antigen. It stands to reason that if our DOTAP/E7 is sufficient to generate such effectors, it must also be capable of initiating proliferation and clonal expansion of such cells. The first evidence to support clonal expansion in response to DOTAP/E7 vaccine treatment came from photographs of the cultures after 21 days of incubation with the vaccine or individual components. As shown in Figure 28, T cells cultured with DOTAP or DOTAP/E7, but not E7 peptide or untreated cultures, increased the number of cells and had visible clumps, indicative of clonal expansion. It is possible that within the untreated and E7 peptide treated cultures, the cells were dying, giving a false positive proliferation result in the DOTAP samples. In the very least, DOTAP is providing a survival signal to the cells. In either case, this data warranted further study and the cultures were then examined for proliferation at a cellular level.

To examine individual cells for proliferation, a dye dilution assay can be used. In this assay, the cell population is labeled with a fluorescent compound. This fluorescence is maintained within the cells during proliferation. The resultant daughters each contain one half of the original fluorescence signal.

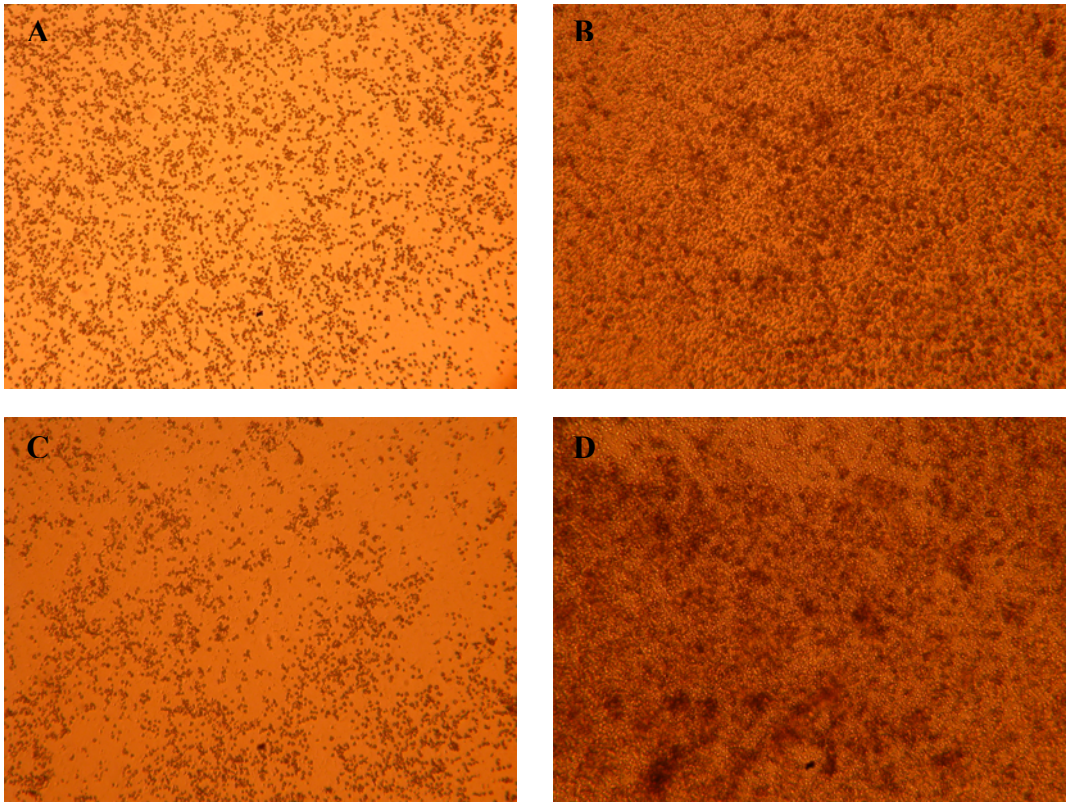


Figure 28. Photographic evidence of proliferation

CD8+ T lymphocytes purified from naïve spleens were incubated in culture in the presence of cytokines (IL-2 and IL-7) and weekly treatments of media only (A), 50uM DOTAP (B), 20uM E7 peptide (C) or 50/20uM DOTAP/E7 vaccine (D). Pictures of the cultures were taken 21 days after first treatment.

When CD8+ T cells are incubated with DOTAP, very few cells take up DOTAP, so most of the cells don't proliferate. In the context of the entire population, the proliferating cells are lost to the non-proliferating background. This is true when looking at several methods of determining proliferation such as both the MTT and dye dilution assays. Indeed, when the CD8+ T cells are labeled with CytoTracker Green and treated with DOTAP/E7, after 6 days in culture, there is no distinguishable difference between the untreated and DOTAP/E7 treated groups as evidenced by the lack of noticeable proliferative peaks (Figure 29).

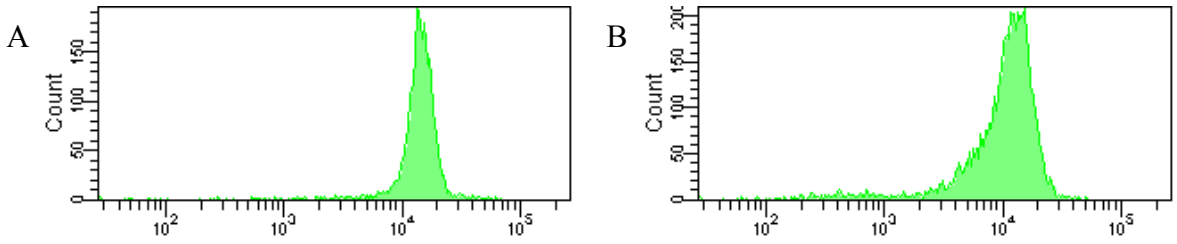


Figure 29. A traditional dye dilution assay for proliferation

Cells were labeled with CytoTracker Green and incubated in the presence of DOTAP/E7 (B) or left untreated (A) for six days in culture in the absence of exogenous cytokine.

Therefore, fluorescent liposomes were used such that it was possible to look at the dye dilution only among the cells that had interacted with the lipid during the first hour of culture, a procedure developed by the author for this application. Freshly purified CD8+ T cells were incubated in the presence of 50uM NBD-DOTAP for 1 hour, washed three times and cultured in the absence of exogenous cytokine with no media changes. On day 6, cells were collected and analyzed by flow cytometry for dilution of NBD into the daughter cells. Propidium iodide exclusion was used to gate living cells, which are plotted on the histogram in Figure 30. Using this procedure, it is possible to see the NBD dilution into the daughter cells with two rounds of replication having occurred in six days. After six days, the parent population consisted of 19.5% of the population. The second and third generations contained 29.5 and 43.6 percent of the total cells, respectively. These results demonstrate that DOTAP is sufficient to cause proliferation in CD8+ T lymphocytes.

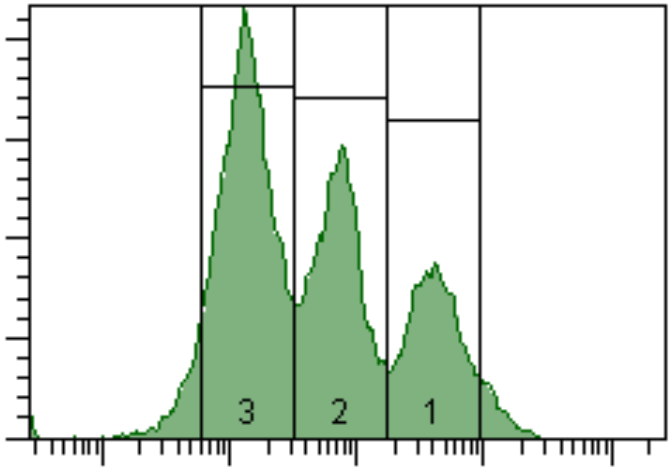


Figure 30. Dye dilution assay for lymphocyte proliferation

Freshly purified CD8+ T cells were incubated in the presence of NBD-DOTAP for 1 hour, washed and cultured in the absence of exogenous cytokine with no media changes. On day 6, cells were collected and analyzed by flow cytometry. Propidium iodide negative cells were gated.

4.3.4 Subsequent signaling events

Knowing that the CD8+ T cells can be activated by, internalize and replicate because of DOTAP, the next goal is to determine if DOTAP can initiate intracellular signaling cascades. Freshly purified CD8+ T lymphocytes were incubated with 50uM DOTAP or PBS (untreated) at room temperature for 5 minutes and immediately fixed with 2% paraformaldehyde. Cells were permeabilized, stained for phosphorylated members of the TCR signaling cascade and analyzed using 4 color flow cytometry. As shown in Figure 31, DOTAP causes an increase in the mean fluorescent intensity of each antibody, suggesting that phosphorylation (ie, activation) of various components of the signaling cascade occurs in rapid response to DOTAP stimulation.

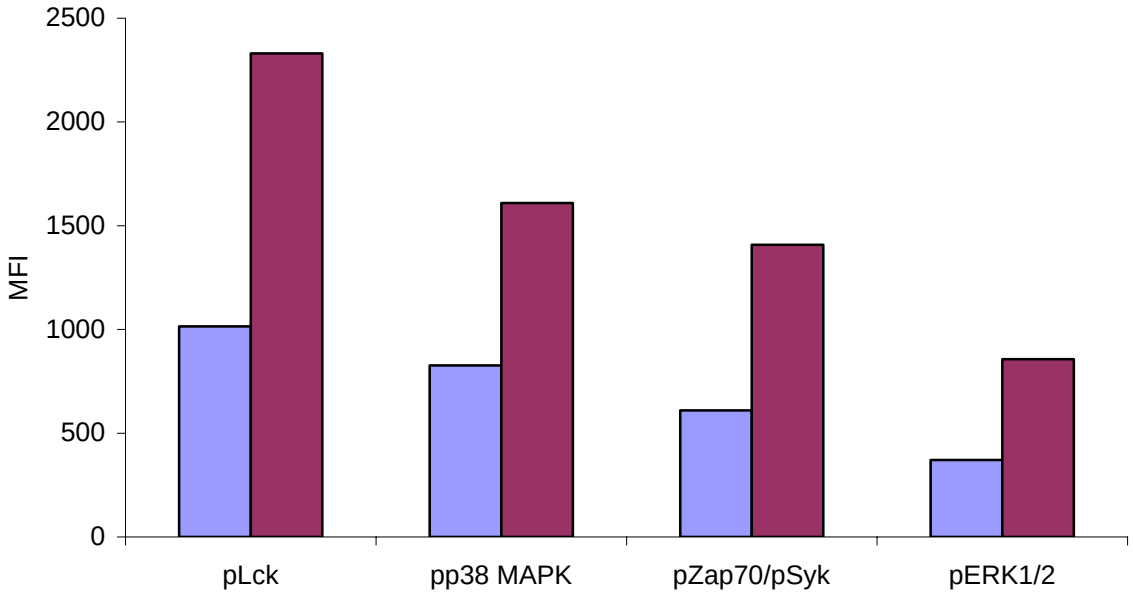


Figure 31. Induction of TCR signaling pathway phosphorylation by DOTAP

Freshly purified CD8+ T lymphocytes were incubated 5 minutes at room temperature in the presence (red bars) or absence (blue bars) of DOTAP before fixation with paraformaldehyde. Cells were then permeabilized and stained for intracellular phosphorylated signaling molecules and analyzed by flow cytometry. Mean fluorescent intensity of each fluorescent antibody is plotted.

4.3.5 Generation of effectors

Since DOTAP/E7 can activate, deliver peptide to and stimulate proliferation of CD8+ T cells, it stands to reason the DOTAP/E7 might interact with the cells to directly generate effectors. In this study, we incubated CD8+ T lymphocytes from spleens of naïve mice with medium only, DOTAP, E7 peptide or DOTAP/E7 vaccine in the presence of IL-2 and IL-7 cytokines. After 2 consecutive 4 day stimulations, all groups were re-stimulated with E7 peptide for 2 days. On day 10, assays were performed to test the specificity and functionality of these cells.

TCR Specificity

Specifically, the cells were tested for TCR specificity by flow cytometry using the DimerX reagent (Becton Dickinson), which is a fusion molecule consisting of an immunoglobulin and 2 MHC molecules (H2D[b] in this case) in a configuration similar to MHC tetramers. The DimerX was loaded with specific (E7) or irrelevant (NP) peptide overnight according to manufacturer's instructions the labeled with secondary antibodies. The cells were stained with each Dimer and analyzed by flow. These results, shown in Figure 32, demonstrate the cells are indeed specific for the E7 peptide. Using the DimerX reagent, cells stain positive for the E7 peptide, regardless of the fluorophore on the secondary antibody, discounting the fluorophore as a possible reason for this positive data.

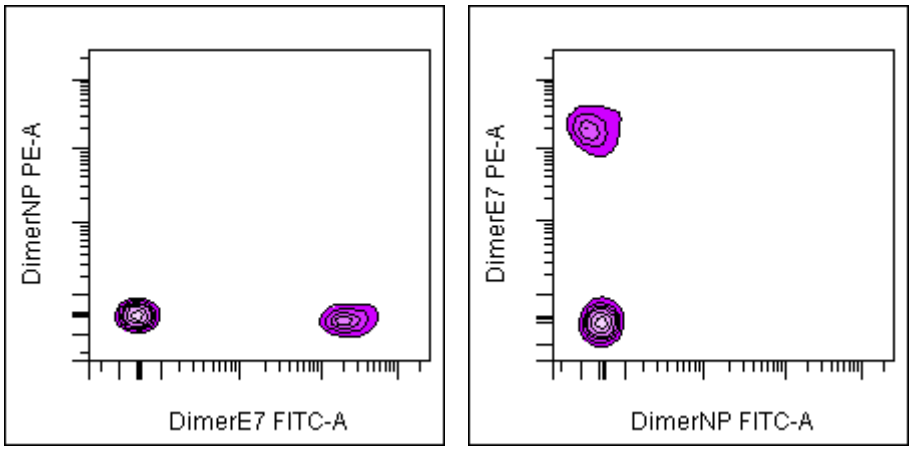


Figure 32. CTL specifically recognize MHC/E7

Cells were stained with CD8 and H-2D[b] DimerX reagent loaded with specific (E7) or irrelevant (NP) peptide and analyzed by flow. CD8+ cells gated.

This experiment was then conducted using only FITC-conjugated secondary antibody for the DimerX reagent. Cells were treated as described above and stained for CD8 (APC) and DimerX (FITC). After the experiment was completed from three separate batches of cells, the data was pooled and analyzed for the percent of cells that were Dimer positive within the CD8+ population. The cells stained with DimerNP and DimerE7 were 0.3% and 38.7% DimerX+/CD8+, respectively. A 2-tailed, unequal variance t test conducted on the pooled data showed the DimerNP and DimerE7 groups were significantly different, with a p-value less than $5x10^{-17}$.

Functional specificity

Furthermore, ELISpot assays were conducted to determine the functionality of the cells as production of IFN-γ correlates with CTL effector functionality [31]. ELISpot assays allow sensitive determination of cytokine-producing cells without the large number of cells needed for a CTL killing assay. Untreated or DOTAP/E7 treated CD8+ T lymphocytes at day 10 of culture were incubated 24 hours in the presence of irrelevant (NP), specific (E7) or no peptide. IFN-γ production was determined by ELISPOT assay using the Becton Dickinson mouse IFN-γ kit according to manufacturer's instructions, counted using the Immunospot reader (Cellular Technologies Ltd) and plotted as IFN-γ producing cells per million. These experiments were repeated at least 3 times and representative images and quantitative data are shown in Figures 33 and 34, respectively. Statistical analysis (2 tailed unequal variance t-test) revealed the DOTAP/E7 treated group stimulated with E7 (>1000 IFN-γ-producing cells per million) was significantly different from all other groups ($p<0.00003$), which clearly indicates an antigen-specific increase in IFN-γ production.

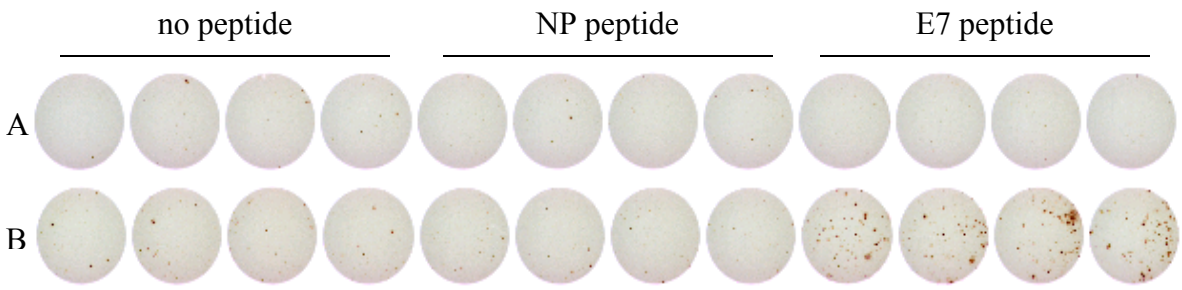


Figure 33. Visualization of IFN-γ ELISpot data

$1.5x10^5$ untreated (A) or DOTAP/E7 (B) treated CD8+ T lymphocytes were incubated 24 hours in the presence of irrelevant (NP), specific (E7) or no peptide. IFN-γ production was determined by ELISPOT assay. Plates were imaged using the Immunospot reader (Cellular Technologies Ltd) and representative images are shown.

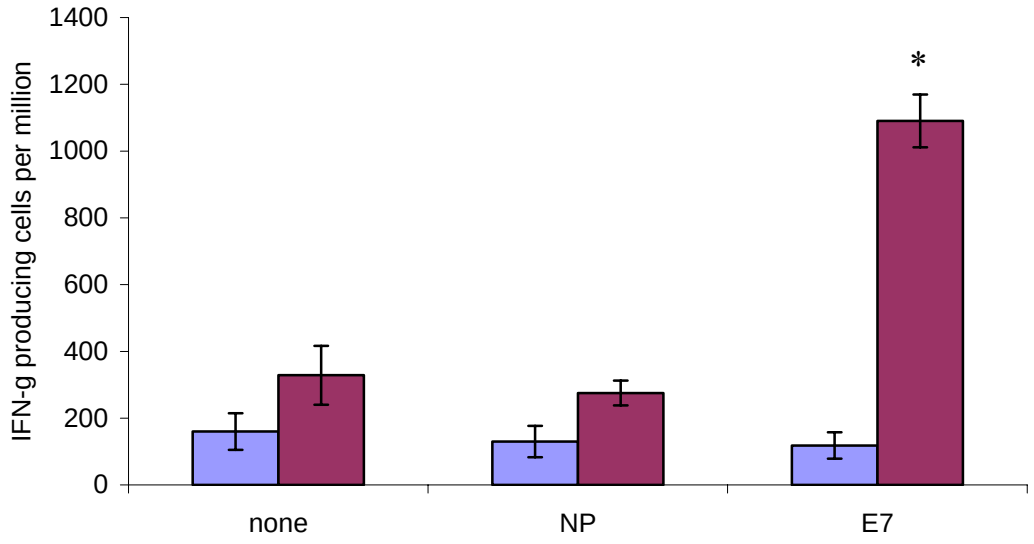


Figure 34. CTL produce IFN-γ antigen-specifically

Untreated (blue) or DOTAP/E7 (red) treated CD8+ T lymphocytes were incubated 24 hours in the presence of irrelevant (NP), specific (E7) or no peptide. IFN-γ production was determined by ELISPOT assay. Spots were enumerated and plotted as IFN-γ producing cells per million. Statistical analysis revealed the DOTAP/E7 group stimulated with E7 was significantly different from all other groups ($p<0.00003$).

In vivo functionality

As discussed, naïve CD8+ T lymphocytes that are cultured in the presence of DOTAP/E7 vaccine possess a T cell receptor specific for the H2D[b]/E7 MHCI/peptide complex and are activated by DOTAP/E7 as shown by the upregulation of CD69 on the surface. A recent publication [31] explained that surface markers alone can not predict TIL anti-tumor activity. Furthermore, the DOTAP/E7 treated CD8+ T cells were shown to have functional specificity for the E7 peptide by means of antigen-specific IFN-γ production, which, according to the same publication [31], correlates with tumor infiltration, an essential component to effector function. Taken together, these are exciting findings, but are moot if the T cells cannot home to the tumor, the location of their effector function. To address this issue, the cultured T cells were introduced to tumor-bearing mice and the tumors visualized the next day for the presence of tumor infiltrating lymphocytes. Specifically, at the conclusion of culture (day 10), DOTAP/E7 treated CD8+ T cells were labeled with AF488SE (green) and mixed with freshly prepared naïve CD8+ T cells that were labeled with AF647SE (red). The cells were mixed at a 1:1 ratio and injected intravenously into TC-1 tumor-bearing mice. Twenty four hours later, tumors were harvested. A cryostat was used to prepare frozen sections (5um) that were mounted with a medium containing DAPI for visualization by confocal microscopy. Images were acquired from different slices and the 3 color overlays are shown in Figure 35. Tumor nuclei were stained with DAPI and appear blue. Naïve and DOTAP/E7 treated CD8+ T cells appear red and green, respectively, in these overlays. Note the DAPI-stained tumor nuclei and green fluorescent, E7-specific CD8+ infiltrating lymphocytes and absence of naïve (red) CD8+ lymphocytes in the tumor sections.

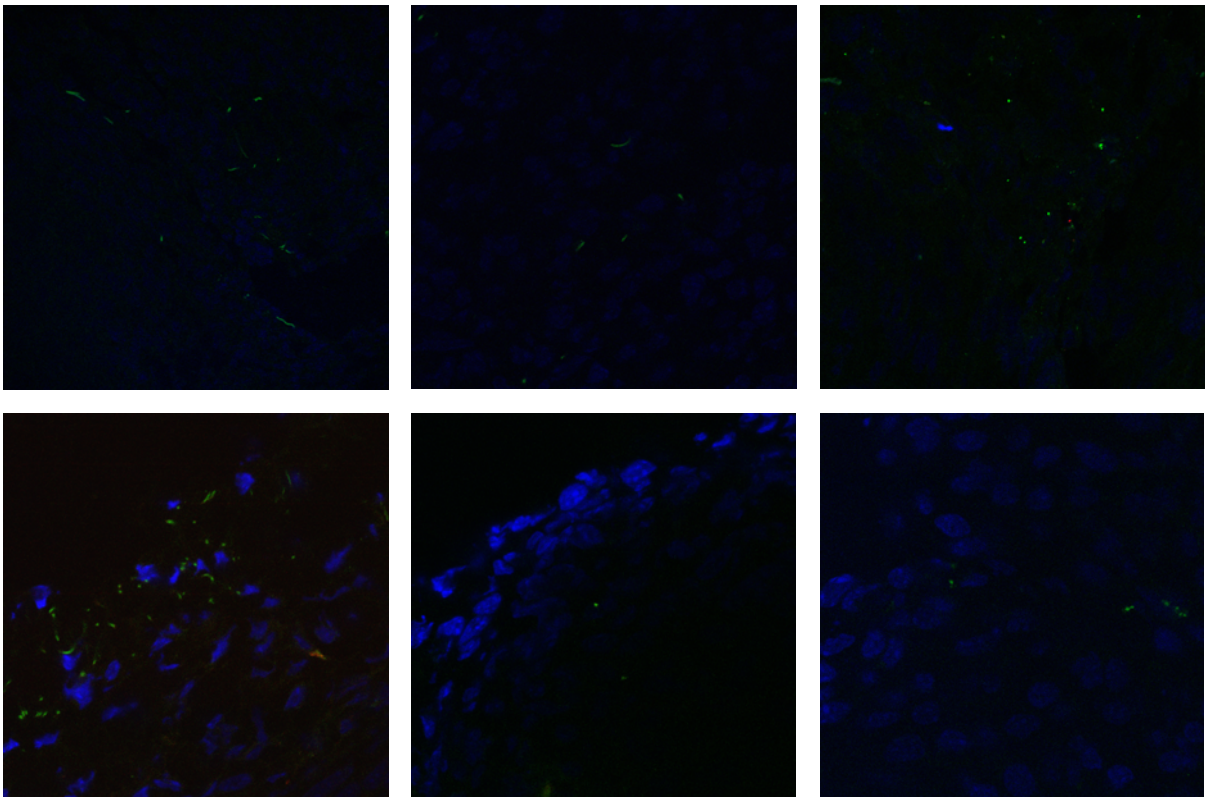


Figure 35. Tumor infiltration

Confocal microscopy images of tumor sections removed from mice 24 hours after naïve (red) or DOTAP/E7 treated (green) fluorescent-labeled CD8+ T lymphocytes were mixed and injected intravenously. Six unique fields are presented.

4.4 DISCUSSION

As shown by an increase in surface levels of the CD69 activation marker, DOTAP/E7 is capable of activating CD8+ T lymphoctyes while simultaneously delivering the peptide cargo to the cells, a result demonstrated using flow cytometry and confocal microscopy. Additionally, during the process of interaction with the cells, DOTAP/E7 has the capacity to stimulate the TCR signaling pathway, a promising step toward explaining the fact that APC are not necessary for effector generation, as determined by an increase in phosphorylated members of the signaling cascade. Furthermore, in the absence of exogenous cytokine, purified CD8+ T lymphocytes cultured in the presence of DOTAP will proliferate in only a few days.

Incubating CD8+ T lymphocytes with DOTAP/E7 resulted in cells with T cell receptors specific for H2D[b]/E7. These cells secrete IFN-γ in an antigen-specific manner and are capable of homing to the tumor environment *in vivo*. Taken together, this data is abundant evidence that the DOTAP/E7 simple vaccine is capable of generating antigen-specific effectors *in vitro* in the absence of antigen presenting cells in a novel pathway of cytotoxic T lymphocyte effector generation. Cytotoxic T lymphocytes (CTL), or effectors, are distinguished based on the expression of CD8 on the cell surface, T cell receptor that recognizes a specific peptide antigen/MHC complex as well as an increase in IFN-γ production upon stimulation by specific antigen.

5.0 DISCUSSION

When the cationic lipid DOTAP (1,2-dioleyltrimethylammoniumpropane) is used to encapsulate an antigenic peptide from the human papillomavirus E7 oncogene (E7), the resultant DOTAP/E7 particles act as a therapeutic vaccine to cause tumor regression through an antigen-specific immune response when the vaccine is injected into mice bearing E7-positive TC-1 tumors. Of critical importance, the DOTAP works as both a delivery vehicle and an adjuvant without induction of a pro-inflammatory cytokine response *in vivo*. We hypothesize that the antigen specific immune response is mediated by dendritic cells *in vivo*. To that end, we investigated the interaction of murine bone marrow-derived dendritic cells (BMDC) with the vaccine *in vitro* in order to recapitulate and more fully understand the process. When BMDC were incubated in the presence of DOTAP or the DOTAP/E7 vaccine, there was a dose and time-dependent upregulation of co-stimulatory molecule expression, indicating that BMDC were activated by the cationic lipid DOTAP. Further experiments indicated that BMDC were capable of internalizing DOTAP liposomes through many endocytotic routes and the vaccine trafficked through the vacuolar pathway. In BMDC that had taken up DOTAP/E7, the peptide localized in perinuclear and defined structures while the DOTAP had a diffuse distribution as imaged by fixed and live cell confocal microscopy. After establishing that BMDC were capable of being activated by DOTAP and examining the internalization and trafficking of the vaccine components, we were interested in validating the antigen processing and presentation. Therefore, we used the indirect

method of presentation wherein we examined the generation of antigen specific effector cells after incubation of CD8+ T lymphocytes, purified from the spleens of naïve mice, with fixed dendritic cells that had been activated by the vaccine. Only if the dendritic cells were capable of antigen presentation should the antigen specific effectors result. Not surprisingly, the DOTAP/E7 therapeutic vaccine was capable of initiating the generation of effector CD8+ T lymphocytes *in vitro* through a dogmatic mechanism, which requires dendritic cell activation and processing of the peptide antigen before the dendritic cells are able to teach the naïve T lymphocytes. Interestingly, we found that the same simple vaccine is capable of generating antigen specific effectors *in vitro* in the absence of antigen presenting cells in a novel pathway of effector generation. Effectors were distinguished based on the expression of CD8 on the cell surface, T cell receptors that recognize a specific peptide antigen/MHC complex as well as an antigen-specific IFN-γ production. Further studies showed that DOTAP could deliver peptide and caused both activation and proliferation of these T cells, revealing a possible mechanism for this novel pathway. Taken as a whole, these observations may lead to additional applications of DOTAP both *in vitro* and *in vivo* to modulate the immune response toward the correction of a variety of diseases.

5.1 SUMMARY OF RESULTS

5.1.1 LPD interaction with dendritic cells

Dendritic cells are mediators of adaptive immunity, allowing the body to specifically recognize and eliminate non-self antigens. It has previously been demonstrated that our cationic lipid LPD based vaccine formulation (LPD/E7) can stimulate an anti-tumor response in a mouse model. This occurs through the initiation of an immune response via TLR activation. Coupled with delivery of a tumor-specific peptide, the immune system eliminates the tumor cells bearing the peptide in order to cause tumor regression.

Dendritic cells are activated by LPS, LPD nanoparticles, and liposomes. Dendritic cells, whether resting or activated, are capable of internalizing liposomes and LPD nanoparticles but not free DNA.

Contrary to accepted wisdom, activated dendritic cells can internalize LPD nanoparticles, even showing an increase in uptake over resting dendritic cells. The reason behind this observation remains unclear. After using BMDC for microscopy, it seems unlikely that differences between primary dendritic cells and DC2.4 dendritic cells are the cause. It is possible that properties unique to LPD are involved. It is also possible these observations are due to inherent properties of the dendritic cells. The latter possibility initially seemed unlikely, considering the dogma of endocytosis down regulation upon DC activation, but other published work has verified the immediate upregulation of endocytosis upon DC activation [9].

Whether flow cytometry or fluorescent microscopy is used to analyze internalization, a negative response can not be argued. If the results indicate a positive response, or internalization, one may argue that fluorescent particles are merely attaching to the surface of the cells and are not, in fact, internalized. To address this issue and study the internalization in greater detail, confocal microscopy was used to visualize thin sections of the cell interior as opposed to the compound images obtained with a traditional fluorescent microscope.

Similar to earlier findings, internalization of LPD particles increased with cellular activation and coincided with an increase in the number of acidic vesicles (endosomes and lysosomes) in the cells. Additionally, in activated dendritic cells, the DNA portion of LPD nanoparticles appears to localize in endosomes and lysosomes. This is particularly interesting because the DNA core of the LPD mimics the peptide component of the simple DOTAP/E7 therapeutic vaccine, which is necessary for the induction of an antigen-specific response. Furthermore, the DNA core traffics through the endosomes, presumably where the unmethylated CpG motifs interact with TLR9 to activate the cells in a non-specific manner.

5.1.2 DOTAP/E7 interaction with dendritic cells

In the absence of immune system activation by TLR stimulation (such as with the LPD/E7 vaccine), the vaccine is still capable of eliciting an anti-tumor response in vivo [24]. This simple vaccine, consisting of a single cationic lipid and the immunodominant eitope of the human papillomavirus 16 E7 oncogene (DOTAP/E7), generates antigen-specific cytotoxic T lymphocytes in vivo toward the regression of established tumors bearing the same E7 peptide [24]. It is hypothesized that dendritic cells internalize the vaccine particles to initiate an anti-E7

adaptive immune response and that DOTAP works both as a protective delivery vehicle for the E7 peptide antigen and as a vaccine adjuvant.

The goal of these studies is to elucidate the mechanism of interaction between dendritic cells and the DOTAP/E7 vaccine toward a more comprehensive explanation of the mechanism of vaccine action. To that end, it is imperative to study the internalization and trafficking of the vaccine by dendritic cells.

BMDC activation by the DOTAP/E7 vaccine (and DOTAP alone but not free peptide) is both time and concentration dependent as shown by an upregulation of CD86 and MHCI surface markers. Clearly, there is time dependence because of the time required for upregulation of surface markers. Interestingly, surface MHCI but not MHCII is upregulated in this manner. The mechanism behind this observation remains unclear and warrants further study. Additionally, the fact that the DOTAP/E7 vaccine doesn't upregulate surface MHCII has implications for future applications of DOTAP for modulation of the immune response.

Bone marrow-derived dendritic cells use many endocytosis pathways to internalize the DOTAP/E7 vaccine via an active internalization process. The DOTAP/E7 vaccine delivers the peptide cargo to dendritic cells, which in turn, traffic the vaccine components through the vacuolar pathway for cross presentation of the peptide.

These DOTAP/E7 treated dendritic cells are sufficient to generate specific and functional effectors both *in vitro* (when incubated with naïve CD8+ T cells) and *in vivo*. Incubating naive CD8+ T lymphocytes with DOTAP/E7-treated dendritic cells cause clonal expansion of the effectors. Additionally, these effectors are specific (have T cell receptors specific for H2D[b]/E7), functional (produce IFN-γ) and capable of homing to the tumor environment *in*

vivo. Furthermore, *ex vivo*-treated dendritic cells re-administered to autologous mice are capable of generating functional, E7 specific CTL effectors *in vivo*.

The DOTAP/E7 vaccine functions to both deliver antigenic peptide as well as 'license' the dendritic cells for the development of antigen specific effectors.

5.1.3 DOTAP/E7 interaction with CD8+ T cells

Previous unpublished observations by this group present the possibility that in a human model in vitro, the DOTAP/E7 vaccine causes T cell proliferation and generates CTL in the absence of dendritic cells. These were exciting findings, but without appropriate histocompatible controls, it was impossible to determine if this was experimental artifact or actual antigen-specific responses. Regardless, these findings were cause for further study and the reason for the subsequent experiments. If the generation of effectors *in vitro* using only a simple lipid/peptide system is possible, then potential applications for this technology are astounding.

Particular issues that required attention include the activation potential, the possible mitogenic properties and the signaling initiation caused by DOTAP/E7. Additionally, it was important to re-evaluate earlier ideas that DOTAP/E7 could indeed generate antigen-specific effectors in vitro in the absence of antigen presenting cells in the context of appropriate controls.

As shown by an increase in surface levels of the CD69 activation marker, DOTAP/E7 is capable of activating CD8+ T lymphoctyes while simultaneously delivering the peptide cargo to the cells, a result demonstrated using flow cytometry and confocal microscopy. Additionally, during the process of interaction with the cells, DOTAP/E7 has the capacity to stimulate the TCR signaling pathway, a promising step toward explaining the fact that APC are not necessary for effector generation, as determined by an increase in phosphorylated members of the signaling

cascade. Furthermore, in the absence of exogenous cytokine, purified CD8+ T lymphocytes cultured in the presence of DOTAP will proliferate in only a few days.

Incubating CD8+ T lymphocytes with DOTAP/E7 results in T cells with TCR specific for H2D[b]/E7. These cells secrete IFN-γ in an antigen-specific manner and are capable of homing to the tumor environment *in vivo*. Taken together, this data is abundant evidence that the DOTAP/E7 simple vaccine is capable of generating antigen-specific effectors *in vitro* in the absence of antigen presenting cells in a novel pathway of cytotoxic T lymphocyte effector generation. Cytotoxic T lymphocytes (CTL), or effectors, are distinguished based on the expression of CD8 on the cell surface, T cell receptor that recognizes a specific peptide antigen/MHC complex as well as an increase in IFN-γ production upon stimulation by specific antigen.

5.2 PERSPECTIVES

It is hypothesized that dendritic cells internalize the vaccine particles to initiate an anti-E7 adaptive immune response and that DOTAP works both as a protective delivery vehicle for the E7 peptide antigen and as a vaccine adjuvant. The goal of this project is to validate the hypothesis and recapitulate the process *ex vivo*. Specifically, elucidating the mechanism of interaction between cells of the immune system and the cationic lipid vaccines toward a more comprehensive explanation of the mechanism of vaccine action.

The results of these studies confirm the hypothesis that dendritic cells take up the vaccine and as a result, are licensed, capable of producing antigen specific effectors. During the course of the study, however, it was also made clear that there exist other possible mechanisms of action of the vaccine (Figure 36). Most notably, the DOTAP/E7 vaccine in the absence of APC is able to cause the generation of antigen specific effectors when incubated with naïve CD8+ T cells *in vitro*. Due to the varied nature of the DOTAP interaction with immune cells, other potential applications for DOTAP both *in vitro* and *in vivo* can be imagined in the modulation of immune response toward the correction of a variety of diseases.

The DOTAP/E7 vaccine is capable of licensing dendritic cells whereby E7 peptide (Figure 36, purple circles) is presented by MHCI (blue boxes) in the presence of co-stimulatory molecules at the surface (green bars). Incubation of these licensed dendritic cells with naïve CD8+ T cells results in E7 specific (purple boxes) and functional (purple starburst) CD8+ effector T cells. In the absence of APC, the CTL generated from naïve CD8+ T cells incubated with the DOTAP/E7 vaccine have E7 specific function.

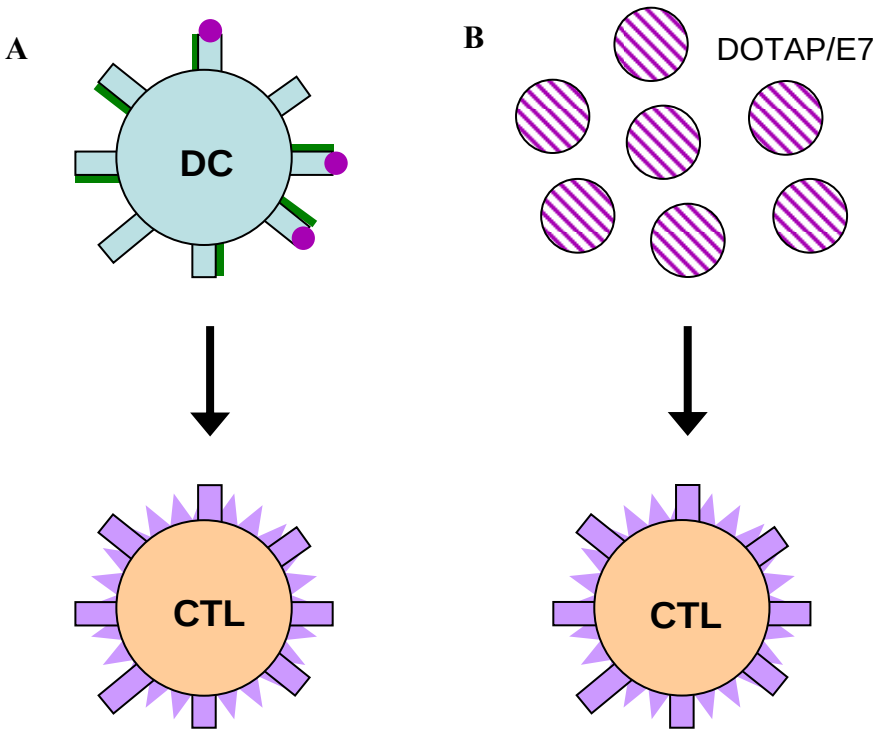


Figure 36. Comparison of pathways to generate antigen-specific effectors

Consistent with the traditional model of effector generation, treatment of dendritic cells with DOTAP/E7 vaccine licenses them for activation of CD8+ T cells and generation of antigen specific effectors (A). Results presented here demonstrate there is a second pathway to effector generation that does not require professional antigen presenting cells (B).

These are exciting findings but need an explanation. It is currently unclear how the CD8+ can become specific and functional effectors in the absence of antigen presenting cells. Figure 37 proposes mechanisms for APC-less effector generation with the traditional model as a comparison (A).

It is possible that CD8+ T cells treated with the DOTAP/E7 vaccine are capable of acting as autologous APC for other naïve CD8+ T cells (B). Although T cells are not professional antigen presenting cells, they do express MHCI and can therefore, be potential antigen

presenting cells. However, after incubation with DOTAP/E7, T cells did not upregulate MHCI on their surface (data not shown). In fact, surface expression of MHCI on DOTAP/E7 treated CD8+ T cells was no higher than background isotype controls. The T cells most certainly express MHCI at low levels on the surface but it is unlikely the surface density is high enough for the T cells to act as an autologous APC. Additionally, it is unclear what provides the proliferation and co-simulation signals. Perhaps the proliferation signal is a soluble signal, such as IL-2 from the autologous APC. The co-stimulation signal could come from the autologous APC or from the DOTAP/E7 directly.

Current studies are underway to determine whether the T cells act as autologous APC or this pathway is bypassed completely. CD8+ T cells will be purified from the spleens of naïve mice that lack MHCI (β2microglobulin knockout strain, C57 background). If the T cells acts as autologous APC, the cells from MHCI deficient mice should be incapable of becoming antigen-specific effectors since this mechanism is Class I dependent.

The other mechanism is one in which the traditional APC pathway is bypassed and the DOTAP/E7 vaccine acts as an APC surrogate (C). In this proposed model, all three required signals (proliferation, specificity, activation) are delivered by the DOTAP/E7 vaccine to the naïve CD8+ T cell. Evidence exists that the DOTAP/E7 vaccine causes proliferation of these cells in the absence of exogenous cytokine. This could be a product of an autocrine response or the vaccine could activate downstream messengers of the IL-2R signaling pathway. This pathway activation could be done directly by the vaccine or could be the results of cross-talk from other activated pathways.

It was also shown the resultant effectors are specific for the E7 peptide. This could be from an engagement of specific TCR by E7 peptide being “presented” by DOTAP or by some

other mechanism after intracellular delivery of the peptide. In the case of TCR engagement, since there is excess, untrapped E7 peptide in the vaccine formulation, it can be assumed there is no free DOTAP. Therefore, cells only see DOTAP in the context of the E7 peptide, explaining how the resultant effectors are 40% E7 specific and not the product of non-specific proliferation.

Additionally, the T cells must be activated in order to become functional effectors. This is normally initiated by the CD3 complex of the TCR in response to prolonged contact of licensed APC and CD8+ T cells. Whether this activation occurs because surface molecules are forced in close proximity by membrane changes after the addition of DOTAP, or if the DOTAP activates downstream members of the TCR signaling pathway directly, is has been shown the TCR signaling cascade is activated by the vaccine. Incubation of the naïve CD8+ T cells with DOTAP causes phosphorylation of members of the TCR signaling cascade such as Lck and Zap70, which are crucial steps in the activation of these cells.

As discussed, data presented here have both supported the original hypothesis (dendritic cells internalize the vaccine particles to initiate an anti-E7 adaptive immune response) as well as produced a new hypothesis. Taken as a whole, substantial evidence is presented here supporting the idea that DOTAP/E7 acts as an APC surrogate for the production of specific and functional effectors from naïve CD8+ T cells in the absence of professional antigen presenting cells. These findings have broadened the scope of the functions of the cationic lipid DOTAP and warrant future study.

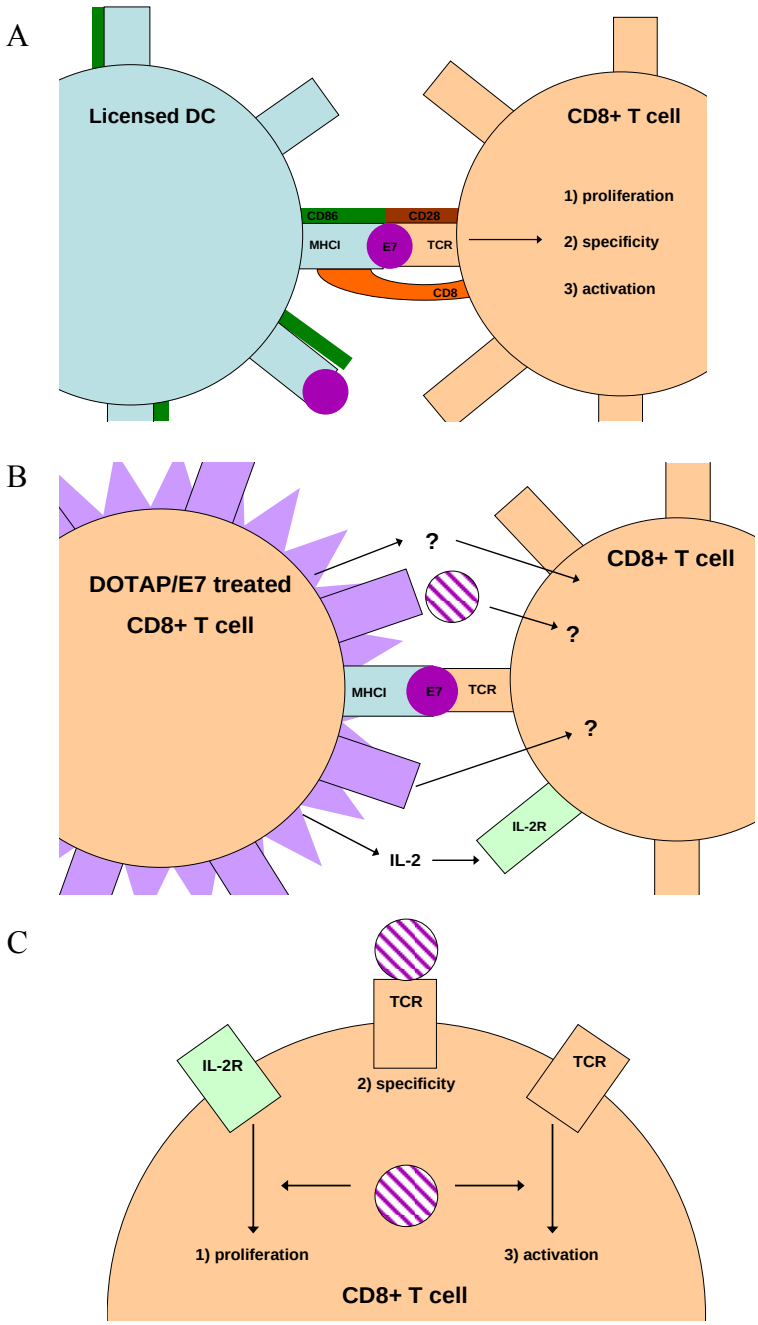


Figure 37. Proposed mechanism of APC surrogacy

The traditional model of effector generation (A), which requires antigen presenting cells is compared with the proposed mechanisms for effector generation in the absence of professional APC: autologous APC (B) and APC surrogacy (C).

5.3 FUTURE DIRECTIONS

This study presents a fairly comprehensive review of the interaction of the DOTAP cationic lipid vaccines with dendritic cells *in vitro*. Of course, more work can always be done. It would be interesting to determine if there is involvement of lipid signaling pathways upon treatment with DOTAP. The cells internalize a large amount of DOTAP cationic lipid and it stands to reason this could alter membrane and intracellular chemistry, leading to such things as altered cellular signaling. Also, it would be beneficial to optimize the technical issues needed to directly examine peptide presentation by the dendritic cells. The story presented here is not complete without this data. The use of inhibitors for the various pathways described here (vacuolar, MHCI recycling, etc.) during treatment with DOTAP/E7 could yield valuable findings regarding the more specific details of the mechanism. Furthermore, a more detailed study of the finding that DOTAP causes upregulation of MHCI and not MHCII on bone marrow-derived dendritic cells could prove highly valuable and have implications for the use of DOTAP to treat certain infectious diseases that are presented through the MHCII pathway. Lastly, the use of dendritic cells deficient in cross-presentation ability (CD8a-DC from Batf3-/- mice) could add valuable support to the *in vivo* dendritic cell hypothesis as well as verify the necessity for the cross-presentation pathway in dendritic cells.

The other major result of this study is the finding that naïve CD8+ T cells treated with the DOTAP/E7 vaccine become specific and functional effectors. Can these cells cause tumor regression in an adoptive transfer model? Can this result be extrapolated to other model systems (different cancers or viruses) or organisms (human)? Through what pathways and mechanisms does the DOTAP/E7 vaccine cause proliferation, specificity and activation in the T cells? Can the generation of antigen specific effectors in the absence of APC be recapitulated in a mouse

model where different cellular compartments are missing (CD11c knockout by diphtheria toxin and/or macrophage knockout mice)?

Is the presence of antigen specific tumor infiltrating lymphocytes indicative of antigen specificity or function? Will the cells still home to an E7 positive tumor if the cells were treated with DOTAP/NP? Will DOTAP/E7 effectors home to an E7 negative tumor? Will the fact that the T cells are functional cause them to home to any tumor, regardless of antigen expression or specificity?

Whether generated through a conventional or novel mechanism, adaptive immunity, as a hallmark, has a memory component. If some of the effector cells (E7 specific T cells) are unable to survive long-term in culture or when re-introduced into mice (CFSE tracking) then perhaps this is not a complete adaptive immune response. Additionally, these cells should have an enhanced immune response when re-stimulated at a later time with the E7 peptide.

These are all questions that remain unanswered and warrant further study. The future goal is improvement of the efficacy and expansion of the function of the DOTAP cationic lipid vaccines.

APPENDIX

DEVELOPMENT OF A PREVENTATIVE DENDRITIC CELL-BASED INDUCIBLE DNA VACCINE USING HPV-POSITIVE TUMORS IN A MOUSE MODEL

Dendritic cells are critical for the induction of both humoral and cellular immune responses. To most effectively capitalize on their abilities, including increased efficacy of nonviral vaccines, the biology of dendritic cells must be fully understood. The goal of this project is the direction of the transgene expression in dendritic cells toward an adaptive immune response against the E7 protein. Inducible plasmids were constructed that express the E7 protein of the human papillomavirus (HPV) 16, an E7/LAMP fusion protein or a reporter protein upon ligand induction using the RheoSwitch system. Time course studies were used to monitor the creation of an antigen pulse and a tumor challenge study in mice was used to functionally confirm antigen presentation by dendritic cells *in vivo*.

The CMV (cytomegalovirus) immediate early enhancer and promoter region is more commonly known as the CMV promoter and is used widely as a strong promoter for mammalian gene expression. This promoter has low expression levels in dendritic cells most likely due to its dependence on NF-κB (the CMV promoter has 4 NF-κB binding sites). It has been demonstrated that dendritic cells must be activated in order to express transgene regulated by the

CMV strong promoter with a high efficiency [32]. Consequently, it is reasonable to imagine the CMV promoter is not necessarily the best choice for gene expression in dendritic cells.

Dendritic cells can become tolerized to tumor-associated antigens (TAA) with prolonged exposure to the antigens. The goal of this inducible vaccine is the generation of an HPV16 E7 gene expression pulse, which should cause an "antigen pulse" and, therefore, maximal presentation by the dendritic cells. This pulse would also complement the priming and boosting schedule employed by many vaccination strategies. The RheoSwitch™ (created by RheoGene, Inc and licensed by NEB BioLabs) inducible promoter system will be employed, which induces gene expression by administration of exogenous chemical ligand. This promoter system has tight regulation and rheostatic control that is dependent on the ligand concentration. This ligand inducer has an effective function at <10nM and has no "crosstalk" or pleiotropic effects, making it an ideal candidate for *in vivo* clinical applications. This inducible promoter system has two components, each carried on a separate plasmid. Plasmid pRG1015 constitutively expresses two proteins under control of ubiquitin promoters that dimerize to form the RheoCept™ protein receptor. This receptor binds to the promoter region of the second, inducible plasmid (Table 3) and upon induction by ligand, activates transgene expression. This tight regulation should allow for gene expression at a high level for a short period of time. The ligand used by RheoGene can be administered orally and would prove most beneficial in regions where access to medical personnel is limited.

Table 3. Expression Construct Details

Construct	Name	Transgene Expression	Plasmid Backbone	Transgene
CMV-SEAP	CS	Constitutive	pCI-neo	SEAP Reporter
TTR-SEAP	TS	Inducible	pG6-TTR	SEAP Reporter
Hygro-SEAP	HS	Inducible	pG6-TTR + Hygromycin^r	SEAP Reporter
CMV-E7	CE	Constitutive	pCI-neo	E7 (HPV 16)
TTR-E7	TE	Inducible	pG6-TTR	E7 (HPV 16)
Hygro-E7	HE	Inducible	pG6-TTR + Hygromycin^r	E7 (HPV 16)
CMV-E7/LAMP	CL	Constitutive	pCI-neo	E7/LAMP1
TTR-E7/LAMP	TL	Inducible	pG6-TTR	E7/LAMP1
Hygro-E7/LAMP	HL	Inducible	pG6-TTR + Hygromycin^r	E7/LAMP1

Plasmid generation

Initial studies were completed using a SEAP (secreted embryonic alkaline phosphatase) reporter gene. When expressed, this protein is secreted from the cell and can be collected in the supernatant (*in vitro*) or in the blood (*in vivo*). Another advantage of this reporter is its heat resistance. After samples are collected, they are heated to 65°C to denature all endogenous alkaline phosphatase and reduce background enzymatic activity. To briefly explain the reporter system, when the SEAP is mixed with the appropriate substrate, the SEAP cleaves a phosphate group to reveal a luminescent compound. The intensity of this light is measured in a luminometer and is directly related to the quantity of SEAP. After initial work was completed with the SEAP reporter gene, experiments were conducted using expression of the HPV16 E7 protein. To increase presentation of the E7 protein to the immune system, the protein will be presented by both MHCI and MHCII molecules. Antigenic peptides of the E7 and Sig/E7/LAMP-1 proteins should be presented predominantly through the MHC I and MHC II

pathways, respectively [33]. The HPV16 E7 gene and the Sig/E7/LAMP-1 cassette, which is the E7 protein flanked by lysosomal associated membrane protein (LAMP) signals, were removed from the pTCsigE7/Lamp1 plasmid [33].

The genes were cloned into plasmids under the regulation of the constitutive CMV promoter or the inducible RheoSwitch promoter, with and without a hygromycin resistance gene (See Table 3). All nine expression plasmids were constructed and verified by DNA sequencing. Representative plasmids (drawn using VectorNTI software) are shown in Figure 38.

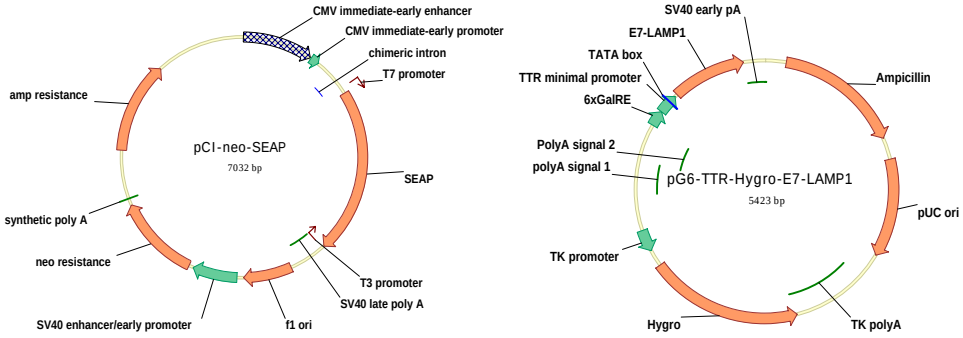


Figure 38. Representative plasmid constructs

These plasmids are representative of the plasmid backbones and transgenes used to construct the plasmids listed in Table 3, drawn using VectorNTI software.

Direct transgene expression in dendritic cells

Preliminary experiments were conducted using the easily transfected HEK 293 cells before proceeding to DC2.4 and BMDC. Moreover, the focus is gene expression, not delivery so it was important to transfect the cells with a proven method. Therefore, the cells were transfected with each of the above constructs using gene gun transfection [32] or Lipofectamine 2000.

Initial experimentation showed that HEK293 or DC2.4 cells transfected by pRG1015 and pTTR-SEAP with Lipofectamine are inducible (Figure 39). Specifically, cells were transfected with both plasmids and cells were induced with 50nM ligand 4 hours post-transfection. Supernatant was collected 24, 48 and 72 hours after induction and analyzed for SEAP expression. As shown in Figure 39, HEK293 cells transfected with seap reporter plasmids by Lipofectamine 2000 expressed large amounts of SEAP in response to 50nM ligand. Additionally, DC2.4 cells transfected by the same method showed low levels of reporter gene expression, as expected, but indicated both gene transfection and expression induction.

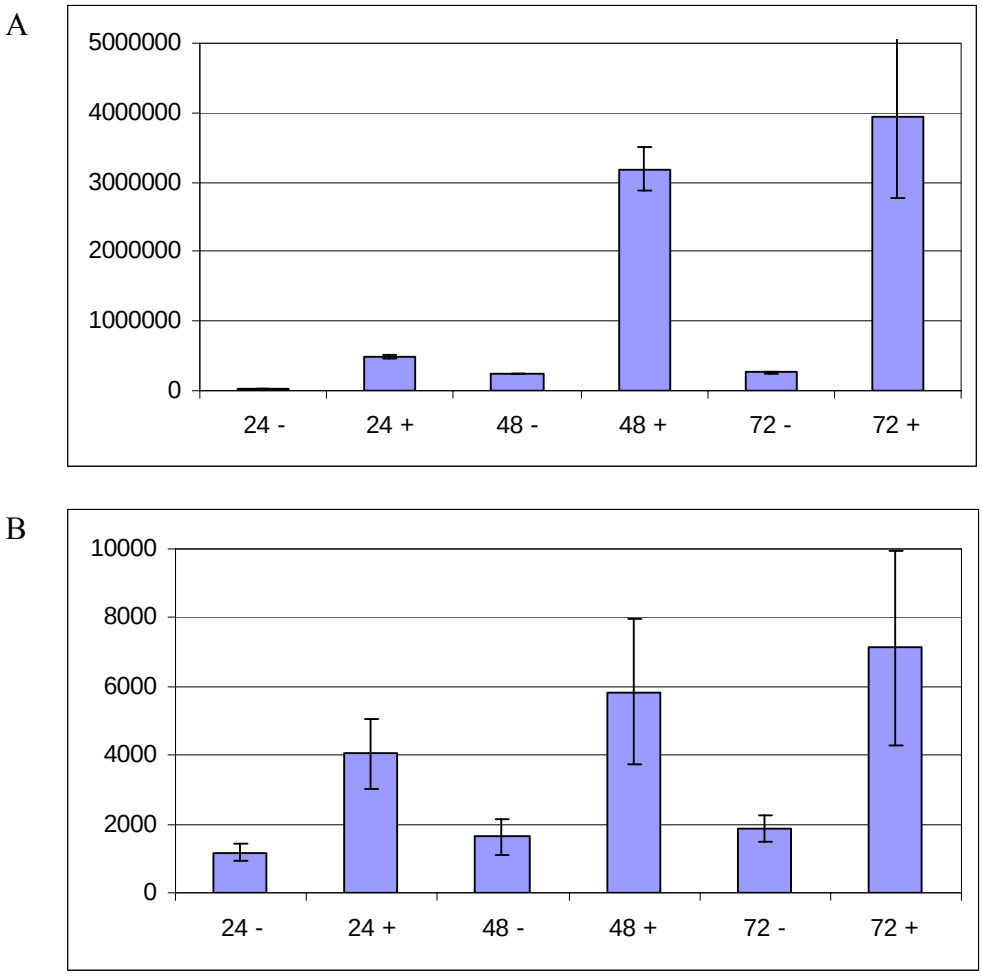


Figure 39. Transgene expression is inducible

HEK293 (A) or DC2.4 (B) cells were transfected with plasmids pRG1015 and pTTR-SEAP using Lipofectamine 2000 and induced with 50nM ligand 4 hours post-transfection. Supernatants were collected 24, 48 and 72 hours after induction and assayed for SEAP production. Luminescence was recorded as counts per second (cps).

Use an inducible promoter to regulate gene expression in DCs

After preliminary work showed that all 3 SEAP reporter plasmids (CS, TS, HS) were inducible in 293 cells (data not shown), expression was further analyzed in dendritic cells. DC2.4 cells were transfected with plasmids RG and HS (both are necessary for proper induction) using Lipofectamine 2000 and induced with the indicated concentration of ligand 4 hours post-transfection. Supernatants were collected 48 hours after induction and assayed for SEAP production. As shown in Figure 40, SEAP expression can be induced and is dose dependent. The slight decrease in SEAP expression might be a statistical anomaly or might represent a true feature of the curve. In either case, physiologically relevant doses would be no higher than 50nM. The same experiment was repeated using the gene gun (BioRad Biolistic Gene Gun shot with 220psi) as the transfection method (Figure 40) and the results were similar. Transfection of primary dendritic cells with SEAP plasmids by both electroporation and gene gun resulted in nearly background levels of SEAP activity (data not shown).

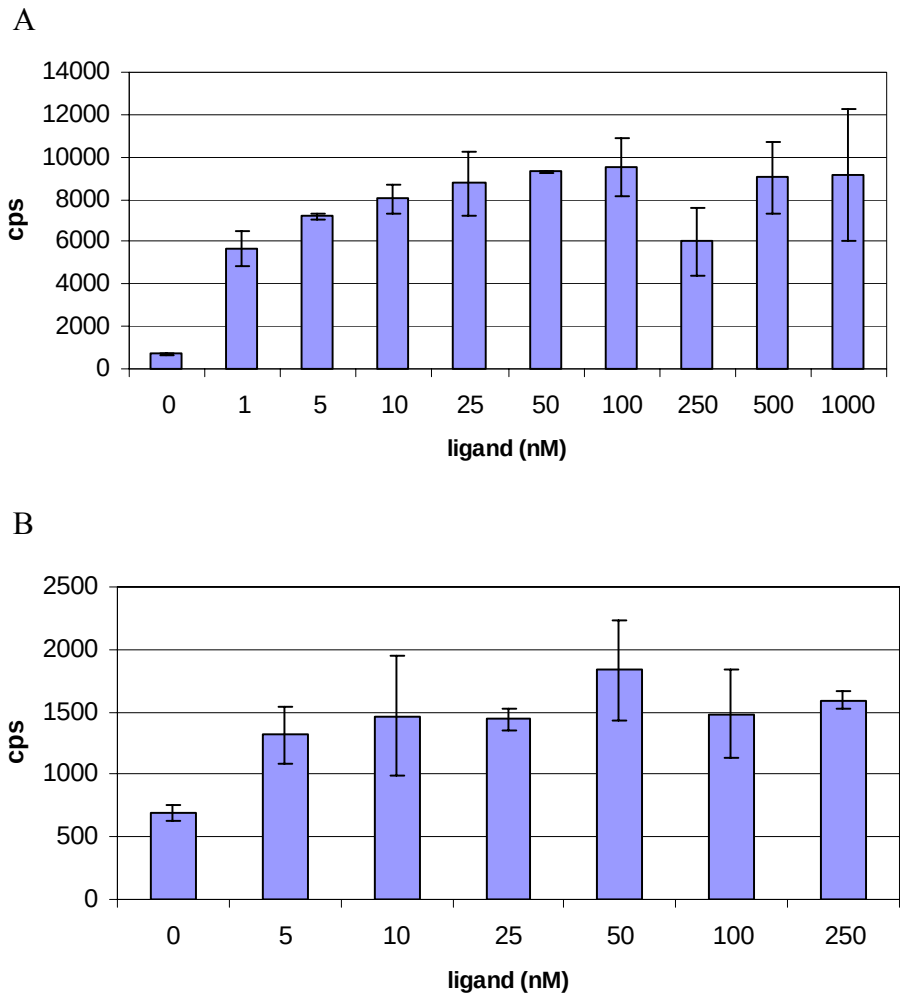


Figure 40. SEAP expression is concentration dependent

DC2.4 cells were transfected with plasmids pRG1015 and pTTR-SEAP using Lipofectamine 2000 (A) or gene gun (B) and induced with the indicated concentration of ligand 4 hours post-transfection. Supernatants were collected 48 hours after induction and assayed for SEAP production. Luminescence was recorded as counts per second (cps).

After positive results for the SEAP constructs, the E7 and E7/LAMP constructs were tested. When induced by the RheoGene ligand, the E7-transfected dendritic cells should produce a pulse of E7 expression and therefore, E7 antigen presentation. Proper E7 gene regulation upon induction by the RheoGene ligand was verified by Western blot in HEK293 (human embryonic kidney) cells, an easily transfected cell line.

HEK 293 cells were transfected with pRG1015 and expression plasmids for E7 (CE, TE, HE) or E7/LAMP (CL, TL, HL) using Lipofectamine 2000 with or without induction by ligand and analyzed by western blot of whole cell lysates 48 hours after transfection. An E7-specific monoclonal antibody was used to detect E7 and a HRP (horseradish peroxidase)–conjugated secondary antibody allowed for visualization on film with a chemiluminescent substrate. As shown in Figure 41, the CMV constructs produced E7 and E7/LAMP regardless of ligand induction. Plasmid TE was inducible with ligand, but no protein was visible upon HE induction. This is most likely due to a problem during the gene expression experiment. Both TL and HL plasmid showed protein expression upon ligand induction.

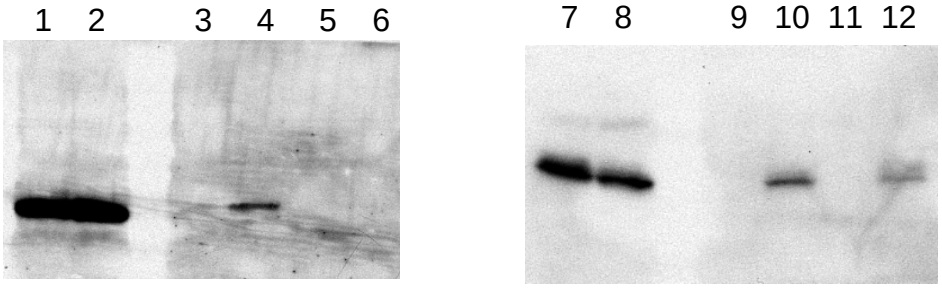


Figure 41. Expression of E7 and E7/LAMP transgenes

HEK 293 cells were transfected with pRG1015 and expression plasmids for E7 (left panel, ~18kDa) or E7/LAMP (right panel, ~21kDa) with or without induction by 50nM ligand and analyzed by Western blot of whole cell lysates 48 hours after transfection. Lanes 1 and 2 (CE), 3 and 4 (TE), 5 and 6 (HE), 7and 8 (CL), 9 and 10 (TL), and 11 and 12 (HL) are un-induced and induced by ligand, respectively. Sixty micrograms of total protein was loaded per well.

For all dendritic cell transfections, procedures were optimized for conditions that caused the least death and highest transfection levels. While it was possible to see transfection in dendritic cells, the level of transgene expression is markedly reduced from levels achieved in other cell types either from a reduction in transfection or expression. Several attempts were made to verify E7 expression in dendritic cells by Western blot, but the signal was undetectable.

Testing in an *in vivo* model

Dendritic cells transfected by gene gun *in vitro* yielded very inconsistent results. Therefore, the more consistent *in vitro* transfection method, electroporation was used for further studies. Since *in vivo* experiments have higher variation than *in vitro* studies, it was decided the use of electrotransfer *in vivo* would yield the most reproducible data.

Although the level of gene expression by dendritic cells may be undetectable by direct methods, very little antigen is required to develop a potent immune response to an antigen. Therefore, a functional (tumor challenge) assay was used to monitor expression. SEAP-transfected cells were used for preliminary experiments to optimize procedures and monitor *in vivo* transgene expression.

Primary dendritic cells were transfected *ex vivo* with the E7 transgene by electroporation and gene gun and reintroduced to syngeneic mice as a preventative vaccine strategy. These immunized mice were challenged with an E7 positive tumor cell line, TC-1 (T.C. Wu, JHU). E7 antigen presentation by dendritic cells was stimulated at various time points by the RheoGene ligand and the effect on tumor size were observed. Unfortunately, no anti-tumor response was evident (data not shown).

To optimize the procedure, mouse muscle cells were transfected *in viv*o by intramuscular electrotransfer to deliver the seap reporter gene. Unfortunately, no circulating SEAP was detected, as the background levels from untreated mice were higher, in most cases, than treated animals.

Intramuscular electrotransfer experiments using E7 plasmids as a traditional DNA vaccine yielded no detectable anti-tumor response upon tumor challenge. Dendritic cells were transfected *in vivo* by both intradermal electrotransfer and gene gun as a preventative vaccine strategy. Unfortunately, no anti-tumor response was evident (data not shown).

In conclusion, although this experimental design had merit, it did not produce positive data. In fact, most vaccinated mice had an increase in tumor growth, perhaps due to tolerance. Many possible explanations exist for why this project failed. Due to the abundance of problems with the execution of this design, the project was abandoned.

BIBLIOGRAPHY

1. Janeway CA, Jr., Travers P, Walport M, Shlomchik MJ (2005) Immunobiology: The immune system in health and disease, 6th edn. Garland Science Publishing, New York, NY

2. Delves PJ, Martin SJ, Burton DR, Roitt IM (2006) Roitt's Essential Immunology, Eleventh edn. Balckwell Publishing Limited, Malden, Massachussets

3. Male D (2004) Immunology, Fourth edn. Mosby, Philadelphia, PA

4. Winzler C, Rovere P, Rescigno M, Granucci F, Penna G, Adorini L, Zimmermann VS, Davoust J, Ricciardi-Castagnoli P (1997) Maturation Stages of Mouse Dendritic Cells in Growth Factor-dependent Long-Term Cultures. Journal of Experimental Medicine 185: 317-328

5. Whiteside TL, Odoux C (2004) Dendritic cell biology and cancer therapy. Cancer Immunology, Immunotherapy 53: 240-248

6. Banchereau J, Steinman RM (1998) Dendritic cells and the control of immunity. Nature 392: 245-252

7. Lipscomb MF, Masten BJ (2002) Dendritic Cells: Immune Regulators in Health and Disease. Physiological Reviews 82: 97-130

8. Austyn JM (2000) Antigen-presenting Cells . Experimental and Clinical Studies of Dendritic Cells. American Journal of Respiratory and Critical Care Medicine 162: 146S-150

9. West MA, Wallin RPA, Matthews SP, Svensson HG, Zaru R, Ljunggren H-G, Prescott AR, Watts C (2004) Enhanced Dendritic Cell Antigen Capture via Toll-Like Receptor-Induced Actin Remodeling. Science 305: 1153-1157

10. Nichols B (2003) Caveosomes and endocytosis of lipid rafts. Journal of Cell Science 116: 4707-4714

11. Liu J, Shapiro JI (2003) Endocytosis and Signal Transduction: Basic Science Update. Biol Res Nurs 5: 117-128

12. Harris J, Werling D, Hope JC, Taylor G, Howard CJ (2002) Caveolae and caveolin in immune cells: distribution and functions. Trends in Immunology 23: 158

13. Le PU, Nabi IR (2003) Distinct caveolae-mediated endocytic pathways target the Golgi apparatus and the endoplasmic reticulum. Journal of Cell Science 116: 1059-1071

14. Orlandi PA, Fishman PH (1998) Filipin-dependent Inhibition of Cholera Toxin: Evidence for Toxin Internalization and Activation through Caveolae-like Domains. Journal of Cell Biology 141: 905-915

15. Smart EJ, Ying Y-s, Donzell WC, Anderson RGW (1996) A Role for Caveolin in Transport of Cholesterol from Endoplasmic Reticulum to Plasma Membrane. Journal of Biological Chemistry 271: 29427-29435

16. Khleif S (2005) Tumor Immunology and Cancer Vaccines. In: Rosen ST (ed) Cancer Treatment and Research Springer, New York, NY, pp. 398.

17. Gregoriadis G (2007) Liposome TechnologyEntrapment of Drugs and Other Materials into Liposomes informa healthcare, New York, New York, pp. 397.

18. Bosch FX, Manos MM (1995) Prevalence of human papillomavirus in cervical cancer: a worldwide perspective. International biological study on cervical cancer (IBSCC) Study Group. Journal of the National Cancer Institute 87: 796-802

19. de Villiers EM (1994) Human pathogenic papillomavirus types: an update. Current Topics in Microbiology and Immunology 186: 1-12

20. Mayordomo JI, Zorina T, Storkus WJ, Zitvogel L, Celluzzi C, Falo LD, Melief CJ, Ildstad ST, Kast WM, Deleo AB, et al. (1995) Bone marrow-derived dendritic cells pulsed with synthetic tumour peptides elicit protective and therapeutic antitumour immunity. Nature Medicine 1: 1297-1302

21. Whitmore MM, Li S, Falo L, Jr., Huang L (2001) Systemic administration of LPD prepared with CpG oligonucleotides inhibits the growth of established pulmonary metastases by stimulating innate and acquired antitumor immune responses. Cancer Immunology, Immunotherapy 50: 503-514

22. Whitmore M, Li S, Huang L (1999) LPD lipopolyplex initiates a potent cytokine response and inhibits tumor growth. Gene Therapy 6: 1867-1875

23. Dileo J, Banerjee R, Whitmore M, Nayak JV, Falo LD, Huang L (2003) Lipid-protamine-DNA-mediated antigen delivery to antigen presenting cells results in enhanced anti-tumor immune responses. Molecular Therapy 7: 640-648

24. Chen W, Yan W, Huang L (2008) A simple but effective cancer vaccine consisting of an antigen and a cationic lipid. Cancer Immunology, Immunotherapy 57: 517-530. DOI 10.1007/s00262-007-0390-4 [doi]

25. Li S, Huang L (1997) *In vivo* gene transfer via intravenous administration of cationic lipid-protamine-DNA (LPD) complexes. Gene Therapy 4: 891-900

26. Ross G, Hubschle T, Pehl U, Braun HA, Voigt K, Gerstberger R, Roth J (2003) Fever induction by localized subcutaneous inflammation in guinea pigs: the role of cytokines and prostaglandins. Journal of Applied Physiology 94: 1395-1402. DOI 10.1152/japplphysiol.00485.2002 [doi]
00485.2002 [pii]

27. Inaba K, Inaba M, Romani N, Aya H, Deguchi M, Ikehara S, Muramatsu S, Steinman RM (1992) Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. Journal of Experimental Medicine 176: 1693-1702

28. Griffiths G, Hoflack B, Simons K, Mellman I, Kornfeld S (1988) The mannose 6-phosphate receptor and the biogenesis of lysosomes. Cell 52: 329-341. DOI S0092-8674(88)80026-6 [pii]

29. Brandes D (1970) Lysosomes in Biology and Pathology. J. T. Dingle and Honor B. Fell, Eds. North-Holland, Amsterdam, and Interscience (Wiley), New York, 1969. Vol. 1, xxiv, 544 pp., illus., $31; vol. 2, xxiv, 668 pp., illus., $37. Frontiers of Biology, vols. 14A and 14B. Science 170: 53-54. DOI 10.1126/science.170.3953.53

30. Basha G, Lizee G, Reinicke AT, Seipp RP, Omilusik KD, Jefferies WA (2008) MHC class I endosomal and lysosomal trafficking coincides with exogenous antigen loading in dendritic cells. PLoS One 3: e3247. DOI 10.1371/journal.pone.0003247 [doi]

31. Oved K, Eden E, Akerman M, Noy R, Wolchinsky R, Izhaki O, Schallmach E, Kubi A, Zabari N, Schachter J, Alon U, Mandel-Gutfreund Y, Besser MJ, Reiter Y (2009) Predicting and controlling the reactivity of immune cell populations against cancer. Mol Syst Biol 5: 265. DOI msb200915 [pii]
10.1038/msb.2009.15 [doi]

32. Larregina AT, Morelli AE, Tkacheva O, Erdos G, Donahue C, Watkins SC, Thomson AW, Falo LD, Jr. (2004) Highly efficient expression of transgenic proteins by naked DNA-transfected dendritic cells through terminal differentiation. Blood 103: 811-819

33. Wu T, Guarnieri FG, Staveley-O'Carroll KF, Viscidi RP, Levitsky HI, Hedrick L, Cho KR, August JT, Pardoll DM (1995) Engineering an Intracellular Pathway for Major Histocompatibility Complex Class II Presentation of Antigens. PNAS 92: 11671-11675