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The Stimulation of Dendritic Cells by Cationic Lipids

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John Peyton Bush, Student

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Dr. Melinda Wilson, Director of Graduate Studies

The Stimulation of Dendritic Cells by Cationic Lipids

THESIS

A thesis submitted in partial fulfillment of the
requirements for the degree of Master of Science in the
College of Medicine
at the University of Kentucky

By

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2019

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ABSTRACT OF THESIS

The Stimulation of Dendritic Cells by Cationic Lipids

The discovery that cationic lipids can independently stimulate the immune system has generated interest in their potential as vaccine adjuvants. Here, we show that the cationic lipid R-DOTAP can independently stimulate type 1 interferon production in dendritic cells in both primary culture and immortalized cell culture. Levels of type 1 interferon production are cell line-dependent and limited in vitro by lipid-induced cell death. We show that cationic lipids can independently activate TLR-7 and TLR-9, suggesting a mechanism for type 1 interferon induction. This TLR-stimulatory activity is not restricted to R-DOTAP and can be extended to other similar cationic lipids in a lipid-specific and TLR-specific manner.

KEYWORDS: Cationic Lipids, Dendritic Cells, Type 1 Interferon, DOTAP, Cancer Vaccine, Cancer

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05/03/2019

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The Stimulation of Dendritic Cells by Cationic Lipids

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CHAPTER 1. INTRODUCTION

As the field of cancer immunotherapy has grown ever more sophisticated, with recent advances in CAR cell engineering[1,2] and immune checkpoint inhibition[3] showing great promise in the clinic, approaches to improve the oldest form of cancer immunotherapy – vaccination – continue to evolve[4]. The first rigorous attempts to artificially induce the immune system to fight cancers date back to the 1890s, when a doctor named William Coley inoculated sarcoma-afflicted patients with streptococcus cultures, based on earlier reports of spontaneous tumor regression in patients who contracted bacterial infections[5,6]. While he observed tumor regression in his patients, the immunological knowledge of the time was not sufficient to explain his results; this was almost two decades prior to Paul Erlich's hypothesis the role of immune involvement in cancer progression[7], and in fact Coley believed that the root cause of cancer could be traced to microorganisms.[6]

With the passage of time came more nuanced and complete understandings of the immune system in ways relevant to immunotherapy. Dendritic cells were famously defined by Steinman and Cohn in 1973[8,9], followed quickly by proof of MHC restriction in 1974[10], although an understanding of peptide binding and structure with relation to MHC would not arise until over a decade later[11]. The existence and mechanisms of CD8+ T cells were elucidated throughout the 1970s through the 1990s, including the function of the thymus in T cell development, the structure of the TCR, and the formation and description of the concepts of T cell activation, tolerance and memory[12].

The basic mechanism of action of vaccines is now fairly well understood[22-26]. Briefly, a vaccine-delivered antigen is taken up by antigen-presenting cells (APCs) such as dendritic cells. Upon activation by pathogen or damage-associated molecular patterns (PAMPS or DAMPS) along with stimulatory cytokines, dendritic cells will activate, mature, and migrate to the draining lymph nodes. Antigen is processed and presented through the exogenous pathway, which presents extracellular antigen to CD4+ helper T cells in the context of MHC Class I molecules, through the endogenous pathway, which presents intracellularly-derived antigen to CD8+ cytotoxic T lymphocytes (CTL) in the context of MHC Class II molecules, or, through the process of cross-presentation, processed through poorly defined pathways such that exogenous antigen is presented through MHC Class I to CD8+ CTLs. This final pathway is what allows a robust CD8+ T cell response against tumors, although the activation of both CD8+ and CD4+ lymphocytes are required to form an efficient, long-lasting immune response against tumors.

From the perspective of modern anti-cancer vaccination, enormous strides were made with the identification of tumor-associated and tumor-specific antigens, beginning in the early 1990s and continuing to the present day. Early attempts at tumor vaccines used autologous and allogenic tumor cells, typically by injection of whole, irradiated tumor cell lysates, but these approaches have largely been left behind in favor of incorporating specific peptides in the vaccine[21]. The first tumor-associated gene to be identified was MAGE-1, discovered in 1991 by Pierre van der Bruggen[13], followed by a number of others, including MART-1[14], gp100[15], NY-ESO-1[16], and hTERT[17]. The broadly recognized difficulty with these early antigens arose from the fact that none of

these genes are specific to the tumor itself. Instead, it is the upregulation of these proteins in cancer cells that allows the breaking of self-tolerance and the establishment of an anti-tumor response, but this also makes obtaining high response rates difficult[20]. In spite of the associated difficulties, the FDA has approved two vaccines for use in the treatment of cancer. The BCG vaccine, approved for the treatment of urothelial carcinoma, can be viewed as a modern extension of the work done by Coley over a century earlier[18]. It is based on the injection of a live, attenuated strain of *Mycobacterium bovis*. Sipuleucel-T (Provenge) has been approved for metastatic castration resistant prostate cancer. As an autologous dendritic cell vaccine that contains on a prostate acid phosphatase-GMCSF fusion protein, Sipuleucel-T is the only approved anti-cancer vaccine that is dependent upon a TAA, and the 4.1-month improved survival time associated with the vaccine unfortunately represents some of the most effective, consistent results that antigen-specific cancer vaccines have been able to provide[19, 20].

In light of the limited efficacy of therapeutic cancer vaccines as single agents, the rarity of the dendritic cell subset responsible for CD8+ T cell priming[27], and the necessity of activating both CD4+ and CD8+ T cells to obtain a robust immune response, much recent work has focused on increasing the efficiency of antigen presentation and delivery.[29-32] Cationic lipids were proposed as a vehicle for DNA transfection in eukaryotic cells in the late 1980s[28], and they have since been expanded as delivery systems for protein[34] and DNA vaccines[33]. Mechanistically, it is understood that the lipid nanoparticle will interact with the cell membrane via electrostatic forces and enter the target cell by an endocytotic, clathrin-dependent process.[35, 36] The lipids fuse with endosomal compartments, where the cationic lipids in the delivery system will both

prevent the degradation of antigen by alkalizing the endosomal pH and destabilize the existing anionic endosomal membrane, resulting in the release of preserved endosomal proteins into the cytoplasm.[37, 38] Cytoplasmic peptides are degraded by the proteasome and processed primarily through the cytosolic pathway to MHC class 1 molecules, which are presented on the surface of the cell. [39, 45] MHC class 1 presentation is responsible for stimulating a CD8+ cytotoxic T lymphocyte response.[45]

More recently, it has been realized that cationic lipids can induce immune responses in an independent and varied fashion. Cationic lipids present in a variety of structures, and it appears that their ability to function as immune stimulators is dependent on structural factors that include the components of the polar head group and length of the carbon chains.[40] A number of cationic lipids in the TAP and EPC families have been shown to induce CD80/86 expression, often used as a marker of maturation in dendritic cells, along with a number of inflammatory cytokines in, but only if the lipids were cationic as opposed to neutral or anionic.[39-41] It has been proposed that cationic lipids act by mimicking the action of exogenous cationic signaling molecules such as arginine and spermine, as well as by modifying the activities of membrane-bound proteins by the disruption of the cell membrane.[43] The cationic lipid diC14-amidine, in addition to the upregulation of CD80/86, induces secretion of IL-12, IL-6, TNF-α, and IFN-β in both human and murine dendritic cell models.[42] Similarly, RPR206252 induces TNF-α, IFN-γ, IL-6 and IL-1β production in human and murine macrophage cell lines, although through a different mechanism[44]. 1,2-dioleoyl-3-trimethyl-ammonium-propane (DOTAP) has been shown to induce the upregulation of CD80/86 via an NF-κB-independent pathway, and to induce the production of a number of cytokines responsible for the activation,

recruitment, and expansion of dendritic cells and CD8+ cytotoxic T lymphocytes in an ERK and PI-3 kinase dependent fashion.[40,41] Interestingly, this includes the downregulation of IL-1β, suggesting a cell-type or lipid-specific immunomodulatory effect. Subsequent work showed that this ERK/PI-3 kinase activation, as well as the upregulation of CD80/86 expression, was dependent on the DOTAP-induced production of reactive oxygen species.[46] Interestingly, the ability of DOTAP to trigger immune responses may be highly conserved – recent work illustrates that DOTAP activates immune responses including ROS production and cell death in plant leaves.[71] The action of DOTAP is enantiospecific; tumor regression assays with R-DOTAP/E7 peptide and S-DOTAP/E7 peptide vaccines have demonstrated that CD8+ lymphocyte activation and tumor regression are found in mice treated with the R-DOTAP-based vaccine, whereas the S-DOTAP-based vaccine only caused a delay in tumor progression and a lack of CD8+ lymphocyte activation.[47]

The diversity of mechanisms of action and downstream outcomes of different cationic lipids suggest that lipid selection in the context of vaccination could be used as a tool to stimulate specific and desirable signaling outcomes and has generated interest with an eye towards rational adjuvant design. Recently, the Woodward lab has described the action of an R-DOTAP-based vaccine containing either MUC-1 peptides or, for use in an HPV tumor system, E7 peptides[57]. The R-DOTAP-MUC1 formulation induced a more robust and polyfunctional T cell response when compared to a Montanide-based control and a control containing Freund's adjuvant, IL-12, GM-CSF and an HBC helper epitope, as measured by IFN-γ, TNF-α, and IL-2 production. In an in vivo, murine, TC1 tumor model, an R-DOTAP based vaccine resulted in infiltration of antigen-specific CD8+ T

cells in the tumor and induced tumor regression, including regression of large tumors. To investigate the increase of polyfunctional CD8+ T cells as a result of RDOTAP administration, we demonstrated the ability of RDOTAP to stimulate cross-presentation in an in vivo model using an adoptive transfer method with CFSE-labeled T cells specific to CD8+ and CD4+ epitopes of OVA such that proliferation of either labeled cell population would require cross-presentation of OVA-derived peptides. Not only did we observe significant levels of cross-presentation, but also noted enlargement of draining lymph nodes. In light of the previously reported immunostimulatory properties of DOTAP and other cationic lipids,[39-44, 46, 47] we injected C57BL6/J mice with R-DOTAP and measured inflammatory gene expression in CD11c+ dendritic cells using Nanostring multiplex analysis. While we observed no increase of NF-κB-dependent cytokines, which is consistent with earlier reports of DOTAP activity[30], there was a marked increase in the upregulation of IFN-α and IFN-β, as well as Stat 1 and CXCL10, both of which are produced in response to interferon production[48,49,57].

The possibility of a targeted induction of type 1 interferons (IFN) by R-DOTAP was intriguing. Type 1 IFN stimulate the cross-priming of CD8+ T cells in viral contexts[54]. In tumor models, Type 1 IFN signaling from the host is required to generate a pool of CD8α+ dendritic cells in draining lymph nodes responsible for CD8+ T cell cross-priming[55]. Type 1 IFN production has also been shown to result in the retention of lymphocytes in lymph nodes through a CD69-dependent mechanism downstream of type 1 IFN signaling.[56] Taken together, this suggests a mechanism for both the enlargement of draining lymph nodes and the polyfunctionality of the T cell repertoire in response to vaccination.

Considering the potential importance of IFN and IFN-related genes in cationic lipid vaccines, we sought to develop an assay suitable for the detection of type 1 IFN in response to cationic lipid stimulation and to elucidate the mechanism of action responsible for that induction. Type 1 IFN can be observed through a reporter cell-based bioassay, expressed in a dose and cell type-dependent manner by BMDCs and immortalized cell lines after R-DOTAP exposure. This exposure is both related to and limited by cytotoxic effects in vitro. R-DOTAP induces type 1 IFN through the activation of the TLR-7 and TLR-9 pathways, in a mechanism that can be extended to other lipids in a structure-specific fashion.

CHAPTER 2. MATERIALS AND METHODS

2.1 Cell Lines

B16-Blue™ IFN-α/β cells (Invivogen, USA) and RAW 264.7 cells (ATCC) were cultured in RPMI medium with 10% FBS, 1mM sodium pyruvate, 1mM L-glutamine, 1x MEM non-essential amino acids, 100 U/ml of both penicillin and streptomycin, 100 µg/mL Zeocin, and 50 µM β-mercaptoethanol (cRPMI).

JAWS II (ATCC® CRL-11904™, USA), were cultured and maintained in Dulbecco's modified eagle medium supplemented with 1mM sodium pyruvate, 1mM L-glutamine, 100 U/mL of both penicillin and streptomycin, and 10 ng/mL recombinant mouse GM-CSF (Biolegend, USA).

HEK-Blue™ Null, HEK-Blue™ human TLR-9, and HEK-Blue™ human TLR-7 (Invivogen, USA) were cultured in Dulbecco's modified eagle medium supplemented with 10% FBS, 100 µg/mL blastocidin, 100 µg/mL Zeocin, 100 U/mL penicillin and streptomycin, and 50 µM β-mercaptoethanol.

2.2 Generation of murine bone marrow-derived dendritic cells

C57BL6/J mice or B6.129S2-Ifnar1tm1Agt/Mmjax (IFNAR-/-) mice (Jackson Laboratories, USA) were euthanized by CO_2 inhalation and cervical dislocation. Tibias and femurs were removed from the animals after sterilization with 70% ethanol and bone marrow flushed into cRPMI. The resulting mixture was passed three times through an 18-

gauge needle to ensure generation of a single-cell suspension and then passed through a 100 μm cell strainer. Cells were counted with a hemocytometer and plated in cRPMI medium supplemented with 5 μg/mL IL-4 and 20 μg/mL recombinant mouse GM-CSF (Biolegend, USA) at a concentration of $2x10^6$ cells per plate. The medium was supplemented on days 3, 5, and 7 after initial plating, and cells were harvested on day 8 for use in experiments.

2.3 Type 1 interferon detection in murine bone marrow-derived dendritic cells

Murine bone-marrow derived dendritic cells (mBMDC) were harvested 8 days after incubation by scraping and seeded in a 96-well plate at a concentration of 200,000 cells per well. RDOTAP dilutions in 280 mM sucrose and LPS dilutions in cRPMI were added at 2x the final concentration, and the plate was allowed to incubate for 18 hours at 37°C, 5% CO_2. After 18 hours, B16 Blue cells harvested with 0.05% Trypsin-EDTA were added to a second 96 well plate at 200,000 cells per well, followed by an equivalent volume of supernatant from the RDOTAP-exposed mBMDCs. A standard curve was generated with recombinant IFN-β in the same plate. The plate was allowed to incubate for 20-24 hours at 37°C, 5% CO_2. 50 μL of this supernatant was then added to 150 μL Quanti-Blue™ detection reagent (InvivoGen, USA), and readings obtained with a spectrophotometer at 650 nm after 3 hours incubation. Analysis was performed using SoftMax Pro 5.3. Statistical significance was calculated with Student's T tests, with p-values of less than 0.01 being considered significant.

2.4 Type 1 interferon detection in cell cultures

RAW 264.7 cells were harvested with 0.25% Trypsin. Type 1 IFN release was detected using the same protocol as above ("Type 1 interferon detection in mBMDCs"). Type 1 IFN release by JAWS II cells was detected in a similar manner, with the exception that RDOTAP was added at 5x final concentration rather than 2x. HEK-Blue Null™, HEK-Blue hTLR-7™, and HEK-Blue hTLR-9™ cells were harvested by tapping the bottom of the cell flasks, seeded in a 96-well plate at 100,000 cells per well, and incubated at 37°C, 5% CO_2 overnight. Media was removed from the well, and 5x concentration lipid dilutions were allowed to sit directly on the cells for 5 minutes before being diluted with cDMEM and incubated for 20 hours. 50 µL supernatant was removed from each well and incubated with 150 µL Quanti-Blue™ detection medium, with readings taken with a Spectramax™ M5 spectrophotometer at 3 hours. Analysis was performed using SoftMax Pro 5.3 and statistical significance calculated using Student's T tests. P-values of less than 0.01 were considered significant.

2.5 Cytotoxicity Assays

JAWS II cells were harvested with 0.25% trypsin and washed with HBSS. $1x10^6$ cells were suspended in 250 µL cRPMI and allowed to incubate with 1 µL NK-TVA™ dye (Cellular Technology Limited, USA) for 30 minutes at 37°C, 5% CO_2. The suspension was washed with DMEM media, spun down at 300xG, and the pellet resuspended in 2 mL DMEM. 10 µL of this suspension + 50 µL cDMEM were added to

each well, for a final count of 5,000 cells per well. Starting readings were obtained using a CTL Immunospot analyzer (Cellular Technology Limited, USA). 50 µL of RDOTAP was added, in triplicate, at 2x final concentration per well, and images obtained at 10 minutes and 2.5 hours.

Lactate dehydrogenase assays were performed using the Cytotoxicity Detection Kit^{PLUS} (LDH) (Roche, Germany). Values were obtained by sampling 25 µL supernatant from the relevant lipid-treated cell type after the full period of incubation, diluting it in 25 µL Dulbecco's PBS, and incubating it at 37°C, 5% CO_2 for 15 minutes, stopping the reaction with 50 µL of the provided stop solution, and taking readings at 450 nm on a spectrophotometer.

CHAPTER 3. RESULTS

3.1 R-DOTAP stimulation induces a type 1 interferon response in murine bone marrow-derived dendritic cells

It has been established that cationic lipids are capable of generating specific immune responses.[30,42,44] In support of this, recent experiments from our lab have demonstrated that IFN-α, IFN-β, and the interferon-related genes CXCL10 and Stat1 are upregulated in mice after injection with R-DOTAP nanoparticles, and that an R-DOTAP-based vaccine induced strong cross-presentation, cross-reactive CD8+ T cell repertoires, and enlargement of draining lymph nodes in immunized mice.[56] To interrogate the role and mechanism of R-DOTAP in generating type 1 IFNs, we generated bone marrow-derived dendritic cells from C57BL6/J mice and exposed them to varying concentrations of R-DOTAP in vitro. We observed a strong dose-dependent response, peaking at 150 μM and dropping off at higher dosages (Figure 3.1A). Previously, we showed that the size of draining lymph nodes and the upregulation of CD69 in mice injected with R-DOTAP was attenuated in IFNαR knockout mice. Therefore, we also generated murine BMDC cultures from an IFNαR knockout strain. IFN production was still observed, again peaking at 150 μM, but was attenuated when compared to wild type C57BL6/J BMDC cultures (Figure 3.1B). Given the demonstrated role of the IFNαR receptor in the production of type 1 IFNs through a positive feedback mechanism and our observations that IFNαR knockout mice did not exhibit the same CD69 upregulation and size increase of the draining lymph nodes in response to R-DOTAP injection, we hypothesize that the IFNα receptor is important in amplifying the production if IFNα in these cultures.

3.2 R-DOTAP stimulates type 1 interferon production in immortalized cell cultures

Although type 1 IFN expression in BMDCs was demonstrable over multiple experiments, it was desirable to develop an immortalize cell culture model to probe the mechanism of R-DOTAP stimulated interferon expression without the individual variation inherent in murine models, and to allow experiments to be performed at a more rapid rate with fewer animal deaths. There have been a number of cell culture lines used to demonstrate specific immune responses to cationic lipids, including RAW-Blue™, DC2.4, TC-1, and transfected human embryogenic kidney (HEK) cells.[41,42,44,46] The RAW264.7 cell line has been previously used to evaluate the immune responses of cationic lipids and lipoplexes.[60,61] Following this precedent, we exposed RAW264.7 macrophage with R-DOTAP nanoparticles for a period of 18 hours and looked for R-DOTAP stimulation using a B16-Blue™ bioassay in a manner similar to the assay used successfully with BMDCs (Figure 3.2A). An observable amount of type 1 IFN was produced, peaking at approximately 150 μM and dropping off at higher dosages, but the levels of interferon produced were low, comparable to those produced by the IFNαR knockout BMDCs. Because a cell line that produced large amounts of type 1 IFN was preferred for the purpose of mechanistic studies, we instead examined the JAWS II murine dendritic cell line using a similar method. We saw a dose-dependent production of type 1 IFN in response to R-DOTAP stimulation, in excess of the amount produced by BMDCs in response to cationic lipid stimulation (Figure 3.2B). Interestingly, JAWS II appeared to require higher levels of DOTAP compared to BMDC, with the response peaking around 450 μM.

3.3 R-DOTAP-induced type 1 interferon production is correlated to and limited by cytotoxicity in vitro

In cell culture models, the R-DOTAP-induced type 1 IFN dose-response curve exhibited a peak response that subsequently decreased at higher doses. We also observed the formation of precipitates and altered cell morphology in wells containing higher doses of R-DOTAP when left overnight. Upon observation under a light microscope, the cell population of these wells appeared to be lysed, with few intact cells and large amounts of cell debris observed at high R-DOTAP concentrations (data not shown). In conjunction with the reported toxicity of cationic lipids[62,63], this led us to suspect that the decrease in type 1 IFN production might be attributable to the death of cells in culture rather than some form of high-dose inhibition. To examine this, we treated JAWS II cells with NK-TVA^{TM} dye, a visual assay in which cells are labeled with a fluorescent dye that is lost upon cell lysis. Dyed cells were treated with different concentrations of R-DOTAP and imaged after 10 minutes and 2.5 hours. Visible cells were counted at each time point and compared to images of the wells prior to R-DOTAP exposure. Population survival was expressed as a percentage of the original population. We observed rapid, concentration-dependent cytotoxicity, with higher doses of R-DOTAP killing over 95% of the cell population within a 2.5-hour time frame (Figure 3.3A). Because the mechanism of cationic lipid fusion with target cells is believed to involve destabilization of the plasma membrane[37], we were concerned that the visual assay might be unreliable; the destabilization could allow release of the fluorescent dye from the target cells, resulting in an artificially high measure of cytotoxicity. To provide a separate measure of

cytotoxicity, we measured cytotoxicity using a lactate dehydrogenase (LDH) assay. While overall cytotoxicity was less than observed with the NK-TVA™ assay, indicating a degree of fluorescent labeling loss due to membrane perturbation, high levels of cytotoxicity were still observed (Figure 3.3C). When combined, the two assays suggest that cell death in response to R-DOTAP exposure occurs rapidly and levels off, with the surviving cells producing the type 1 IFN detected by the bioassay. The loss of type 1 IFN production at higher dosage levels is therefore not necessarily due to a lower level of interferon production per cell, but because fewer cells remain alive to produce it.

3.4 Cationic lipids activate TLR-7 and TLR-9

Type 1 interferons can be produced through the actions of the TLR-9 subfamily of toll-like receptors. It has been demonstrated that cationic lipids will fuse with the endosomes of target cells and destabilize them,[37,38] so we reasoned that R-DOTAP might interact with TLR-9 family receptors to stimulate type 1 IFN production. Because HEK cells modified to overexpress TLRs have been successfully used to demonstrate the dependence of cationic lipid signaling on TLRs[44], we obtained HEK-Blue™ cells modified to express individual TLRs, either TLR-7 or TLR-9, along with a secreted alkaline phosphatase (SEAP) reporter gene controlled by an NF-κB promotor. HEK-Blue™ Null1 cells were used as a negative control. We observed both TLR-7 and TLR-9-dependent SEAP expression in response to R-DOTAP stimulation (Figure 3.4A)[57]. Cationic lipid-induced immune responses have been shown to be dependent on the nature of the lipid head group and carbon tails.[40] To determine if the responses observed in R-

DOTAP-stimulated cells held for other cationic lipids, we stimulated HEK-Blue™ TLR-9, TLR-7, and Null1 cells with the cationic lipid 1,2-di-O-octadecenyl-3-trimethylammonium propane (DOTMA), the cationic lipid 1,2-dioleoyl-sn-glycero-3-ethylphosphocholine (chloride salt) (DOEPC), and the neutral lipid 1,2-Dioleoyl-sn-glycero-3-phosphocholine (DOPC). Consistent with previous reporting with other downstream targets, the neutral lipid (DOPC) was unable to induce a response in either the TLR-9 or TLR-7 expressing cell line (Figure 3.4D). The cationic lipids induced responses; DOEPC produced both TLR-7 and TLR-9 dependent SEAP expression in a dose-dependent manner (Figure 3.4B), while DOTMA more specifically produced TLR-9 dependent responses when compared to the null control line (Figure 3.4C).

A. B.

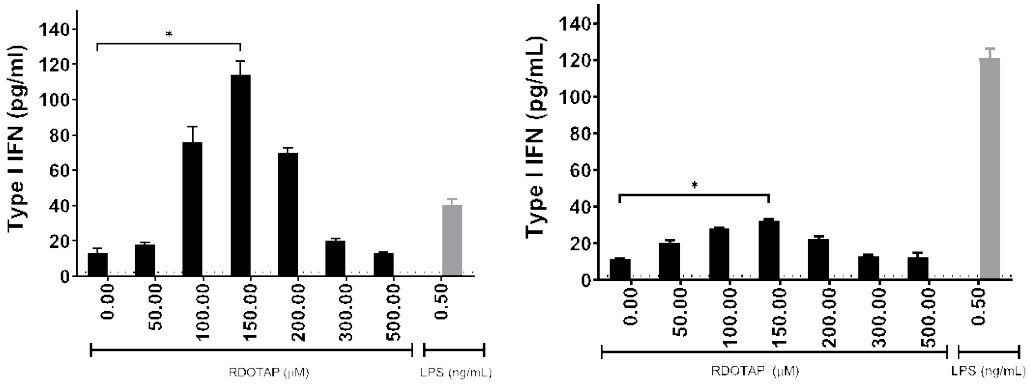


Figure 3.1 Type 1 interferon expression is induced by RDOTAP in BMDCs in a dose-dependent, IFNαR-dependent manner. Murine bone marrow-derived dendritic cells from C57BL6/J (Figure 3.1A) or an IFNαR $^{(-/-)}$ strain (Figure 3.1B) were exposed to varying concentrations of R-DOTAP for a period of 18 hours, and type 1 IFN production quantified with a B16-Blue^{TM} reporter cell-based bioassay. Responses in both cell lines peaked at approximately 150 μM, but the response magnitude in IFNαR$^{(-/-)}$ derived cells was attenuated when compared to the wild type. Asterisks indicate statistical significance ($p<0.01$).

A. B.

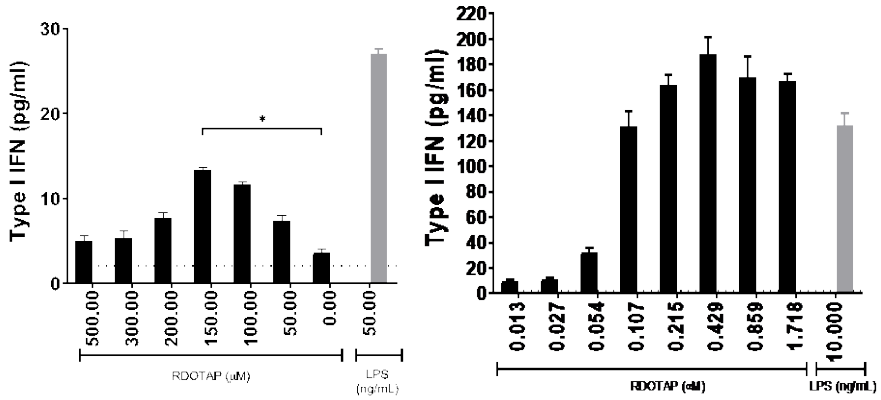
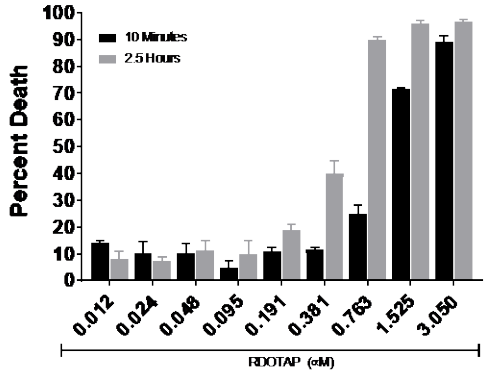


Figure 3.2 R-DOTAP responses in vitro are cell-type dependent. RAW264.7 macrophages (Figure 3.2A) and JAWS II dendritic cells (Figure 3.2B) were exposed to R-DOTAP for a period of 18 hours, and type 1 IFN production quantified with a B16-Blue^{TM} reporter cell-based bioassay. Expression of type 1 IFN was highly dependent on cell type, with RAW264.7 expression comparable to IFNαR$^{(-/-)}$ BMDC expression, and JAWS II expression higher than WT C57BL6/J-derived BMDC expression. Asterisks represent statistical significance ($p<0.01$).

A.



B.

C.

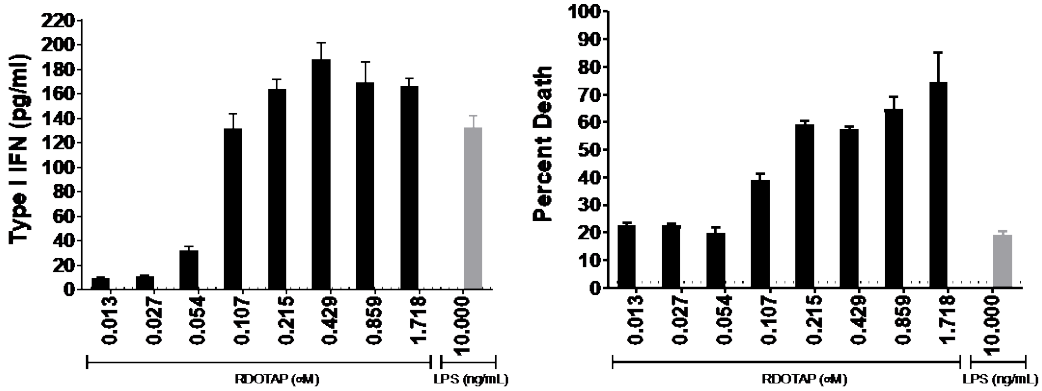
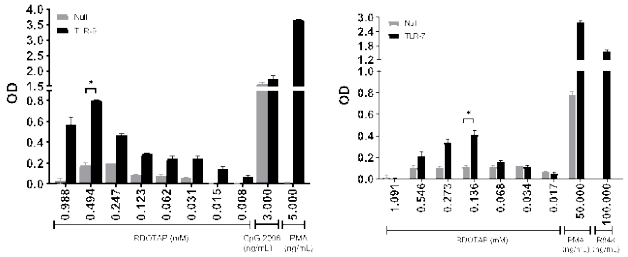


Figure 3.3 Type 1 IFN expression is related to and limited by the cytotoxic effects of R-DOTAP exposure. JAWS II cells were stained with CTL dye and exposed to R-DOTAP. Cells were imaged at 10 minutes and 2.5 hours, and cell death quantified as a percent of initial cell density (Figure 3.3A). JAWS II cells were exposed to R-DOTAP for a period of 18 hours, and supernatant examined using an LDH release assay to quantify cell death (Figure 3.3C). IFN production was quantified from the same plate using a B16-Blue™ reporter cell-based bioassay. JAWS II IFN production is the same experiment as in Figure 3.2B, provided here for ease of reference.

A.



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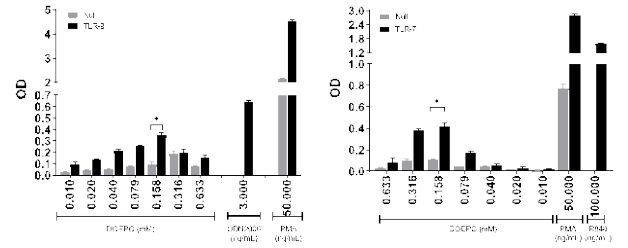
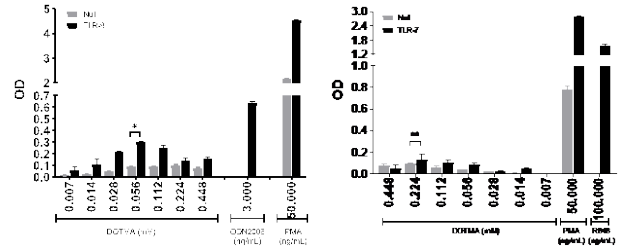


C.



D.

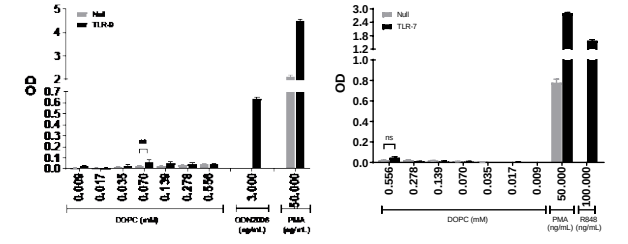


Figure 3.4 Cationic lipids stimulate TLR-9 and TLR-7 in a lipid-specific manner. HEK-Blue^{TM} hTLR9, hTLR7, and Null cell lines were incubated with cationic and neutral lipids for 20 hours and SEAP release quantified by spectrophotometric readings in the presence of a reporter dye. DOPC, a neutral lipid, did not induce either TLR-9 or TLR-7 activity (Figure 3.4D), while the cationic lipids DOEPC and R-DOTAP induced both (Figure 3.4A and B). DOTMA, another cationic lipid, induced TLR-9 activity but not TLR-7 activity (Figure 3.4C). Asterisks represent statistical significance ($p<0.01$).

CHAPTER 4. DISCUSSION

Cationic lipids have been well-established as agents that increase the efficiency of antigen and nucleic acid delivery to target cells.[28,33,34] Previous work indicates that the actions of cationic lipids are linked to structure, specifically the nature of the cationic head group and the carbon chains, but the exact nature of the mechanisms by which they induce responses has yet to be well described.[40,43] The Huang lab has shown that DOTAP upregulates CD80/86 expression on exposed dendritic cells and that it activates PI-3 kinase and ERK, leading to the production of CCL2, CCL3, and CCL4 and the downregulation of IL-1β.[40,41] Further work from the same laboratory indicates that ERK activation is dependent on the production of reactive oxygen species produced in the first 10 minutes of stimulation by DOTAP, providing both a mechanism for cationic lipid-induced chemokine generation, co-stimulatory molecule expression, and apoptosis.[46] We demonstrate that R-DOTAP induces the production of type 1 IFN in both BMDCs and immortalized cell lines in an IFNαR-dependent and cell-type dependent manner. The peak of the response, at 150 μM, corresponds nicely to previous work that puts peak CD80/86 expression in BMDC cultures in response to R-DOTAP stimulation at the same concentration, although given the in vitro cytotoxicity levels demonstrated here and elsewhere[46] this is likely to be an artifact of the assay limitations rather than a reflection of an ideal dose in a live system. Type 1 IFN production suggests an explanation for a number of previous observations about the effects of DOTAP, including the upregulation of CD80/86 expression, the activation of PI-3 kinase, and the expression of the IFN-dependent cytokine CCL2.[67-70] The extent to which IFN activity is connected to ROS-

induced ERK activation remains to be elucidated. The stimulation of type 1 IFN production in immortalized cell cultures provides an avenue to explore the mechanism of action of cationic lipid stimulation without the individual variation present in BMDC cultures. We observed an increased ability to produce type 1 interferon in the JAWS II murine dendritic cell line when compared to BMDCs, while experiments with RAW264.7 macrophages failed to produce IFN at comparable levels to either JAWS II or BMDC cultures. RAW264.7 macrophages have been previously used to describe the stimulatory effects of cationic lipids.[60] The cell line has previously been shown to produce IFN-β mRNA in response to cationic lipid/DNA lipoplex stimulation, although only in a transient fashion early after stimulation[61]. This, coupled with our data, suggests that the immunomodulatory effects of cationic lipids are dependent on cell type, but that the effects can be studied in appropriate immortalized cell lines as well as in BMDC cultures.

Using the JAWS II cell line, we investigated the cytotoxic effects of R-DOTAP exposure on cell culture. Cationic lipid-dependent cytotoxicity has been well reported,[42,62,63] and we suspected that the reduced expression of type 1 interferon in vitro at high dosages of R-DOTAP was a result of this toxicity. We found a dose-dependent increase in cell death upon exposure to R-DOTAP that appears to limit the ability of cells to produce responses in vitro. This suggests limitations to the study of R-DOTAP stimulation using a cell model system, in that the magnitude of the responses elicited could conceivably be inhibited by the death of cells in culture in a manner not reflective of the in vivo reality.

To investigate the mechanism underlying the ability of R-DOTAP to induce type 1 IFN expression, we examined the ability of the lipid to activate TLR-9 and TLR-7 in HEK cells modified to express either TLR-7 or TLR-9 in a specific fashion. The potential of cationic lipids to interact with toll-like receptors has precedent; the ability of the cationic lipid diC14-amidine to induce IFN-β and TNF-α has been tied to stimulation of TLR-4.[42] TLR recognition was specific; TLR-2 and TLR-3 were not shown to be involved in diC14-amidine immunostimulation, and the response in the presence of LPS was attenuated, suggesting a specific recognition of the unsaturated carbon chains of diC14-amidine by TLR-4.[42] Conversely, RPR206252 acts through the PRRs TLR-2 and NLRP-3.[44] Taken together, this suggests that different PRRs can recognize cationic lipids in a structure-specific manner to induce discrete downstream effects. In line with these reports, we observed the stimulation of SEAP production by TLR-7 and TLR-9 in response to R-DOTAP and sought to characterize the responses of structurally related lipids DOEPC and DOTMA. Both DOEPC and DOTMA, like DOTAP, contain quaternary amino head groups and carbon tails of similar length. The effects of the neutral lipid DOPC were examined as a control. DOEPC induced both TLR-7 and TLR-9 mediated SEAP production, peaking around 150 μM in a manner similar to R-DOTAP, while DOPC induced neither a TLR-7 or TLR-9 mediated response. DOTMA induced TLR-9 mediated SEAP production, but did not induce more TLR-7 mediated production compared to a null control cell line. The reason for this specificity in the case of DOTMA is unclear. Differences in immune responses are typically attributed to the structure of the head group and carbon tails[40], and the failure of the neutral lipid DOPC to activate either TLR-7 or TLR-9 in this study supports this. However, DOTMA, R-DOTAP, and DOEPC

all contain similar head groups and carbon tails. DOTMA possesses an ether linker region rather than an ester linker (found in DOEPC and R-DOTAP), and while there do not appear to be any reports that linker regions affect immunostimulatory ability, a structure-dependent model of TLR stimulation allows the possibility of unreported structure-dependent activity.

Another possibility regarding the mechanism of TLR activation is related to the lipid-associated cytotoxic effects demonstrated here and elsewhere.[46,62,63] Consistent with past observations of cationic lipid-dependent cytotoxicity,[42] we observed rapid and extensive cell death as a result of R-DOTAP exposure using both visual fluorescence-based assays and more traditional LDH release assays. It is difficult to separate the release of type 1 IFN from the presence of cell death – we did not observe type 1 IFN production at dosages too low to induce cell death, but whether this is because cell death is required to induce the production of type 1 IFNs or simply because dosage levels were too low to cause either cell death or TLR stimulation is unclear. However, TLR-7 and TLR-9 are both capable of responding to endogenous RNA and DNA, respectively, and it has been known for a number of years that TLR-9 and TLR-7 respond strongly to DOTAP/RNA or DNA lipoplexes[74,75], which raises the possibility that cationic lipids are taking up nucleic acids released from lysed cells and transporting them into endosomes, where they can be recognized by TLR-7 or TLR-9.[35-38]

The finding of lipid-induced IFN production has the potential for use in the clinic. IFN-α has both been investigated and approved by the FDA for the treatment of hairy cell leukemia[50] and melanoma[51], and it has been found effective in a number of other malignancies[52]. IFN therapy has, however, resulted in significant toxicities, including

hepatotoxicity, fatigue, fever, nausea, and depression.[52,53] Because the mechanism of type 1 IFN-mediated anti-tumor activity is predominately dependent on hematopoietic targets rather than the direct response of neoplastic cells,[65] the ability to induce type 1 IFN expression in a targeted manner in physiologically relevant contexts presents an opportunity to avoid toxicities associated with systemic IFN administration.

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