Latent Membrane Protein-1 as a Vaccine Adjuvant

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UNIVERSITY OF MIAMI

LATENT MEMBRANE PROTEIN-1 AS A VACCINE ADJUVANT

By

James Termini

A DISSERTATION

Submitted to the Faculty
of the University of Miami
in partial fulfillment of the requirements for
the degree of Doctor of Philosophy

Coral Gables, Florida

May 2014
UNIVERSITY OF MIAMI

A dissertation submitted in partial fulfillment of
the requirements for the degree of
Doctor of Philosophy

LATENT MEMBRANE PROTEIN-1 AS A VACCINE ADJUVANT

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Dendritic cells are a promising cell type for immunotherapy due to their potent immunostimulatory potential. Nevertheless, improper maturation may account for the limited efficacy of dendritic cells in immunotherapy clinical trials. Latent Membrane Protein-1 (LMP1) of the Epstein-Barr virus was examined for its ability to mature and activate dendritic cells as a gene-based molecular adjuvant. Dendritic cells were transduced with an adenoviral vector expressing LMP1 under the control of a Tet-inducible promoter. LMP1 was found to mature and activate both human and mouse dendritic cells. LMP1 enhanced in vitro migration of dendritic cells toward CCL19, as well as in vivo migration of dendritic cells to the inguinal lymph nodes of mice following subcutaneous injection. LMP1-transduced dendritic cells increased T cell proliferation in a Pmel-1 adoptive transfer model and enhanced survival in a B16-F10 melanoma tumor model. LMP1 also enhanced protection in a vaccinia-gag viral challenge model. Surprisingly, LMP1 generated high levels of IL-12p70 secretion in mouse dendritic cells and failed to induce dendritic cell exhaustion when compared to Mimic maturation. LMP1-transduced human dendritic cells were able to secrete IL-12p70 and TNF alpha in response to restimulation. In contrast, Mimic matured DC were impaired in IL-12p70 secretion following restimulation. We propose that LMP1 is a promising molecular adjuvant for dendritic cell therapy, able to mature and activate dendritic cells while allowing IL-12p70 secretion in response to restimulation in the draining lymph node.
ACKNOWLEDGMENTS

First, I would like to express my appreciation for my mentor, Dr. Geoffrey Stone. His dedication and commitment to science has inspired me to become a better scientist. His selfless mentorship has helped me gain the experience and expertise necessary to succeed in my field. I would also like to thank my committee: Dr. Eckhard Podack, Dr. Eli Gilboa, Dr. Samita Andreansky and Dr. Margaret Fischl, for all their critiques and guidance. They have trained me how to think critically as a scientist. Their comments and criticisms have made my thesis what it is today.

I am greatly honored to have Dr. Jacques Banchereau as my external examiner. I couldn’t think of a person more perfect for the role and I appreciate him taking the time to serve as my examiner.

I would like to further extend my gratitude to Dr. Ligou Niu, previous post-doc in the lab, for his willingness to train me when I first arrived. Also, Sachin Gupta and Saravana Kanagavelu, post-docs in the Stone Lab. Without their collaboration, assistance, and friendship, I could have never accomplished so much.

Last but not least, I would like to thank my amazing family, who has always been there to support me. They have always pushed me to succeed. I hope my future accomplishments and discoveries will make them proud.
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<th>Description</th>
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<tr>
<td>APC</td>
<td>Antigen presenting cells</td>
</tr>
<tr>
<td>BCR</td>
<td>B cell receptor</td>
</tr>
<tr>
<td>BMDC</td>
<td>Bone marrow-derived murine dendritic cells</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CBA</td>
<td>Cytometric bead array</td>
</tr>
<tr>
<td>CCL</td>
<td>Chemokine C-C motif ligand</td>
</tr>
<tr>
<td>CCR7</td>
<td>C-C chemokine receptor type 7</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony forming units</td>
</tr>
<tr>
<td>CpG</td>
<td>Cytosine-phosphate-guanine</td>
</tr>
<tr>
<td>CTAR</td>
<td>C-Terminal Activating regions</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic T lymphocyte</td>
</tr>
<tr>
<td>CXCR4</td>
<td>C-X-C chemokine receptor type 4</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cells</td>
</tr>
<tr>
<td>DC-SIGN</td>
<td>Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin</td>
</tr>
<tr>
<td>DDC</td>
<td>Interstitial/dermal DC</td>
</tr>
<tr>
<td>Dox</td>
<td>Doxycycline</td>
</tr>
<tr>
<td>EBV</td>
<td>Epstein-Barr Virus</td>
</tr>
<tr>
<td>ELISPOT</td>
<td>Enzyme linked immunospot</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
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<tr>
<td>GM-CSF</td>
<td>Granulocyte macrophage colony stimulating factor</td>
</tr>
<tr>
<td>HAART</td>
<td>Highly active antiretroviral therapy</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>---------</td>
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</tr>
<tr>
<td>IFN-α</td>
<td>Interferon alpha</td>
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<td>IFN-β</td>
<td>Interferon beta</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon gamma</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IRES</td>
<td>Internal ribosomal entry site</td>
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<tr>
<td>IRF7</td>
<td>Interferon regulatory factor 7</td>
</tr>
<tr>
<td>JNKs</td>
<td>c-Jun N-terminal kinases</td>
</tr>
<tr>
<td>LC</td>
<td>Langerhans cells</td>
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<tr>
<td>LMP1</td>
<td>Latent Membrane Protein-1</td>
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<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>LTNPs</td>
<td>Long-term nonprogressors</td>
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<tr>
<td>MAP Kinase</td>
<td>Mitogen-activated protein kinase</td>
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<tr>
<td>MCP-1</td>
<td>Monocyte chemoattractant protein 1</td>
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<tr>
<td>mDC</td>
<td>Myeloid DC</td>
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<tr>
<td>MFI</td>
<td>Mean fluorescence intensity</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MLR</td>
<td>Mixed leukocyte reaction</td>
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<tr>
<td>MoDC</td>
<td>Monocyte-derived dendritic cells</td>
</tr>
<tr>
<td>MOI</td>
<td>Multiplicity of infection</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>p38</td>
<td>P38 mitogen-activated protein kinases</td>
</tr>
<tr>
<td>PAMPs</td>
<td>Pathogen-associated molecular patterns</td>
</tr>
<tr>
<td>PAP</td>
<td>Prostate acid phosphatase</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Form</td>
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</tr>
<tr>
<td>PBL</td>
<td>Peripheral blood lymphocytes</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>pDC</td>
<td>Plasmacytoid DC</td>
</tr>
<tr>
<td>PFU</td>
<td>Plaque forming units</td>
</tr>
<tr>
<td>PGE2</td>
<td>Prostaglandin E2</td>
</tr>
<tr>
<td>PI3 Kinase</td>
<td>Phosphatidylinositol-4,5-bisphosphate 3 kinase</td>
</tr>
<tr>
<td>Poly I:C</td>
<td>Polyinosinic: polycytidylic acid</td>
</tr>
<tr>
<td>PRRs</td>
<td>Pattern recognition receptors</td>
</tr>
<tr>
<td>SEAP</td>
<td>Secreted embryonic alkaline phosphatase</td>
</tr>
<tr>
<td>SFC</td>
<td>Spot-forming cells</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TES2</td>
<td>Transformation effector site 2</td>
</tr>
<tr>
<td>TLRs</td>
<td>Toll-Like Receptors</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor alpha</td>
</tr>
<tr>
<td>TPA</td>
<td>Tissue plasminogen activator</td>
</tr>
<tr>
<td>TRADD</td>
<td>TNFR1 associated death domain protein</td>
</tr>
<tr>
<td>TRAF</td>
<td>Tumor necrosis factor receptor associated factor</td>
</tr>
<tr>
<td>Treg</td>
<td>T regulatory cells</td>
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1.1 Overview of Dendritic Cells Biology

Dendritic cells (DC) were first described by Ralph Steinman in the 1970’s [1]. Dendritic cells are potent antigen presenting cells (APC) that play a central role in both the innate and adaptive immune responses, and are able to induce both immunity and tolerance [2, 3]. They act as sentinels scanning the body for foreign antigens. Once an antigen is encountered, DC will process and present the antigen in the form of peptide-MHC complexes to naïve T cells. These T cells will then differentiate into different types of antigen-specific T cells with diverse functions [4]. The nature of the T cell response generated by a DC depends not only the DC subset but also the stimulus it receives during maturation. Responses range from a proinflammatory Th1 response that generates CTL based immunity, a Tfh response that generates humoral immunity, or a tolerogenic Treg response [5, 6].

Being at the center of the innate and adaptive immune systems, dendritic cells express a wide variety of pattern recognition receptors (PRRs). PRRs are primitive innate receptors that recognize components of foreign pathogens or pathogen-associated molecular patterns (PAMPs) [7]. PRRs expressed by DC include Toll-like receptors (TLRs). TLRs are conserved throughout evolution as far back as Drosophila. TLRs recognize antigens such as Lipopolysaccharide (LPS) of bacteria, unmethylated CpG DNA, single and double stranded RNA [7]. Expression of TLRs varies depending on the subset of DC examined. However, monocyte-derived dendritic cells (MoDC), used for dendritic cell immunotherapy are known to express TLR1-6, TLR8, TLR10 and low levels of TLR7 [8]. When a DC detects a microbial antigen via a TLR, it responds by
upregulating costimulatory molecules such as CD80/CD86 and secretion of proinflammatory cytokines such as TNF-α, IL-6, and IL-12p70 [9].

Only a small number of DC are necessary to potentiate a robust immune response [5, 10]. The main role of dendritic cells is to capture, process, and present antigens to lymphocytes. Dendritic cells undergo maturation after antigen capture and PRR ligation or other stimulatory signals. Maturation involves the acquisition of certain abilities critical to dendritic cell function. These include the presentation of antigen on the cell surface via MHC, and the down regulation of further antigen processing. Matured DC also increase the expression of surface costimulatory molecules such as CD80/86 and CD40 and chemokine receptors such as CCR7 and CXCR4 that allow migration to the draining lymph node (Fig. 1.1). Finally, DC acquire the ability to secrete proinflammatory cytokines such as TNF-α and IL-12p70 [4]. While in the lymph node or spleen, DC present antigen to T and B cells, costimulate these cells, and secrete cytokines to initiate an antigen-specific immune response [5]. Dendritic cells can directly present antigen to B cells, either whole antigen or antigen fragments bound to the DC surface. DC possess degradation pathways for antigen processing and MHC presentation, as well as non-degradation pathways used to present antigen to B cells for B cell receptor (BCR) engagement [11]. For activation of the T cell, antigen must be taken up by the DC, degraded, and presented via MHC I or II [5].

There are many different subtypes of DC recognized today. The more these subsets are studied, the more we realize the diverse abilities each possess. In humans, dendritic cells are broken down into two categories; myeloid DC and plasmacytoid DC (mDC and pDC respectively). These DC vary depending on whether they are found in
the tissue or circulating in the blood. Human dendritic cells found in the blood can be
classified by the expression of three different cell-surface molecules: CD303, CD1C, and
CD141 [3, 12]. CD303+ pDC are known for their ability to secrete IFN-α and rapidly
initiate a CD8+ T cell response when viral infection is detected. It is believed that pDC
are the most important cell type for antiviral innate immunity. pDC are known to
modulate the activity of B cells, T cells, natural killer cells, and other myeloid derived
DC [13].

Myeloid DC (mDC) can be divided into two further subsets based upon whether
they are found in the skin or the blood. In the blood, mDC include three DC subsets
known as CD1C+ DC, CD141+ DC, and CD16+ DC. As their name suggests, CD141+
DCs express CD141 on their surface [14]. They have been shown to be effective at
capturing antigen and cross presenting the antigen on MHC class I [15]. This makes
them an important DC subset for the generation of antigen-specific CD8+ T cell immune
responses. CD1C+ DC are known for their expression of a number of TLRs and their
superior ability to stimulate naïve CD4+ T cells. However, CD1C+ DC are very poor
cross-presenters and do not stimulate strong CD8+ T cell responses [14, 15]. CD16+ DC
are known to have weak antigen presenting abilities. However these DC are capable of
eliciting a strong proinflammatory immune response [16].

In the skin, there are two additional mDC populations, epidermal Langerhans
cells (LC) and interstitial/dermal DC (DDC). Langerhans cells are distinguished by their
expression of CD207 while DDC are distinguished by the expression of DC-SIGN
(CD208) [8, 17]. LC are found in the epidermis while DDC reside in the dermis. When
there is an inflammatory event, whether infection or injury, both LC and DDC respond to
PAMPs and locally secreted cytokines, inducing migration of these DC to the local lymph nodes [8]. Importantly, similar to CD141+ DC, Langerhans cells are known to be exceptional at cross-presenting antigens to CD8+ T cells via MHC I [3, 8, 15]. This presentation is key for the development of CD8+ CTL. CD14+ DC are known for their ability to stimulate a Tfh response and drive humoral immunity [16].

Sallusto et al. were the first group to demonstrate that dendritic cells could be differentiated from CD14+ monocytes purified from the blood [18]. Using GM-CSF and IL-4, they were able to culture dendritic cells that were capable of antigen capture, processing, and presentation. These cells are what we now commonly refer to as monocyte-derived dendritic cells (MoDC). MoDC most closely resemble interstitial/dermal DC [8]. The Sallusto method of DC differentiation continues to be the standard protocol used for the majority of DC-based immunotherapies [19]. MoDC can be generated from both mouse and human monocytes and are a powerful tool to study dendritic cell biology. After they are generated, MoDC can be matured by a variety of cytokines or costimulatory molecules. The most commonly used maturation cocktail for MoDC is the Mimic cytokine cocktail (Mimic). Mimic is composed of TNF-α, IL-1β, IL-6, and PGE2. Mimic was first described by Jonuleit et al. as a “mimic” of monocyte conditioned media able to fully mature DC [20]. The reasoning behind the use of Mimic is to enhance the effect of TNF-α by adding pre-inflammatory cytokines [21]. In theory this should mimic the microenvironment where DC maturation typically occurs.

Other protocols have also been developed for the maturation of MoDC. These protocols range from 2-7 days to generate a fully mature DC and contain a variety of other stimulatory molecules such as LPS, CD40L, and IFN-γ. Maturation protocols have
even been developed that mature a DC in situ using adjuvant pretreatment of the injection site. Some of the more successful protocols are described in Section 1.5.

Overall, it has been shown that MoDC are effective antigen presenting cells and can be matured to express high levels of co-stimulatory surface receptors. Upon stimulation, MoDC secrete pro-inflammatory cytokines that can drive a robust Th1 response [3, 22, 23]. However, DC maturation is complex. It appears that different maturation protocols can lead to different functional abilities of MoDC [10]. A key
question for the research community is which maturation protocol will lead to the best therapeutic DC.

1.2 Dendritic Cell:T Cell Interactions

When interacting with T cells, dendritic cells can present antigen via MHC I or MHC II (Fig. 1.2). For proper T cell activation there are 3 key signals that a DC must provide. These signals determine the type of T cell generated, as well as the activation state of the T cell [24]. The first signal is antigen presentation via MHC:TCR interaction. The second signal is costimulation via co-receptors. Costimulatory molecules such as CD80/86 can engage and stimulate CD28 on the T cell surface. Similarly, CD40 on the DC surface can interact with CD40L on the T cell, providing co-stimulatory signals to the DC (Fig. 1.2). The third signal is proinflammatory cytokine secretion, such as IL-12p70 [25]. During DC:T cell interaction, the DC and T cells receive stimulation via MHC II: TCR engagement. This engagement along with DC costimulation via CD80/CD86 triggers IFN-γ release by the T cell and CD40L on the T cell binds to CD40 on the dendritic cell. CD40 ligation together with IFN-γ triggers the secretion of large amount of IL-12p70 by the dendritic cell [26, 27]. IL-12p70 is a key proinflammatory cytokine that is necessary for the activation and differentiation of the T cell. IL-12p70 enhances T cell proliferation and differentiation of CD8+ T cells into cytotoxic T lymphocytes (CTL) [28].
As discussed previously, certain subsets of dendritic cells are excellent at capturing antigen and cross-presenting that antigen on MHC I to stimulate CD8+ T cells [3, 8]. Although other antigen presenting cells (APC) such as macrophages and B cells can cross-present, depletion studies have indicated that DC appear to be the main APC responsible for cross-presentation in vivo [29]. During microbial infection, uninfected DC typically cross-present antigen taken up from neighboring infected cells, leading to robust CD8+ T cell activation [15]. Although all cells express MHC I, mature dendritic cells are required to generate a robust immune response. Other cell type may present
antigen via MHC I but lack the costimulation and cytokine production necessary for a properly primed T cell response [2, 5].

In the absence of proper maturation, DC will fail to induce an inflammatory immune response, and instead induce tolerance [4, 30]. It is now known that this process serves an important immunological function. Most auto-reactive T cells are deleted during thymic development. However, some auto-reactive T cells escape to the periphery. By presenting self-antigen in the absence of maturation, DC maintain peripheral tolerance via the deletion of self-reactive T cells or by inducing the differentiation of regulatory T cells (Tregs) [4, 6, 31]. Similarly, immature DC are known to lack costimulatory molecule expression, secrete low levels of cytokines, and retain the ability to capture and present large amounts of antigen. These features allow immature DC to present antigen without stimulating an inflammatory T cell response [32]. Huang et al. have nicely demonstrated this process. They showed that immature DC frequently uptake and present self-antigens. They also demonstrated that immature intestinal DC frequently contained antigen from apoptotic cells and that these DC migrate to T cell areas of the lymph node and induce tolerance [33].

1.3 Dendritic Cell Immunotherapy for Cancer

The goal of immunotherapy for cancer is to generate antigen-specific anti-tumor CTL to fight established tumors and also to generate lasting immunologic memory to help control tumor relapse [3]. Due to the potent immunostimulatory capacity of dendritic cells, ex vivo generated DC are an attractive reagent for this strategy [34]. Only a small number of DC are necessary to potentiate a robust immune response [5, 10].
practice, ex vivo DC are generated from a patient’s peripheral blood, typically from CD14+ monocytes, loaded with different types of antigens, matured, and injected into the patient [22]. To date, there have been numerous dendritic cell clinical trials for cancer immunotherapy. Overall, DC therapy has been found to be safe and well tolerated. In addition, trials have demonstrated that both CD4+ and a CD8+ T cell responses can be generated against tumor antigens [35-40]. Although DC immunotherapy increases antigen-specific circulating T cells, DC immunotherapy clinical trials have only shown limited clinical efficacy to date [3, 10, 35, 37-40]. There appear to be inconsistencies between the immune responses observed in the blood and the overall clinical response [22]. Methods for predicting clinical efficacy are a topic of much research interest.

Currently, there is only one FDA approved dendritic cell therapeutic vaccine. Sipuleucel-T (PROVENGE) is approved as an immunotherapy for men with metastatic castration-resistant prostate cancer [41, 42]. PROVENGE is designed to induce immune responses against prostate acid phosphatase (PAP). A fusion protein of PAP and GM-CSF is used as the antigen for this immunotherapy. The PAP-GM-CSF fusion protein is incubated with PBMC enriched for APC and the cells are then administered to the patient by intradermal injection. In clinical trials median survival increased by 4.1 months compared to a placebo control. 3 year survival also increased from 21.7% to 31.7%. [41, 42]. This study highlights the potential for clinical benefits for DC immunotherapy.

Dendritic cell immunotherapy is also being tested for a variety of other cancers using a number of techniques for DC generation, antigen loading, and DC maturation (Tab. 1.1). Clinical trials have shown partial efficacy in many cancers including melanoma, glioma, and renal cell carcinoma [36, 37, 39, 43, 44]. There are several
different types of antigens used for DC immunotherapy for cancer. These include peptide antigens, mRNA encoded antigens, and whole tumor lysate. Many studies are attempting to determine which form of antigen is likely to generate the best immune response for particular types of cancer [3, 22, 45]. It is believed that DC transfected with antigen mRNA are superior to other antigen loading strategies at generating an immune response [24]. However, mRNA is not applicable for all types of cancers. In general, mRNA is most useful when tumors express well-defined tumor associated antigens. DC therapy is
also being combined with other forms of cancer therapy for a number of ongoing DC immunotherapy clinical trials. These include tumor resection by surgery, chemotherapy, tumor radiation, and other cancer therapeutics such as monoclonal antibodies [3, 35, 46]. These approaches are likely to improve the efficacy of DC-based cancer therapies.

1.4 Dendritic Cell Immunotherapy for HIV

Based on promising results from cancer immunotherapy, DC therapy has also been explored as a treatment option for chronically HIV infected patients. During chronic HIV infection, a patient’s immune system is unable to eradicate the virus from the body. However, certain individuals termed long-term nonprogressors (LTNPs) have a unique ability to control viremia. Studies have shown that viral control by LTNPs is dependent on a population of polyfunctional HIV-specific CD4+ and CD8+ T cells [47, 48]. Due to the important role of dendritic cells in stimulating and sustaining polyfunctional HIV-specific T cells, several groups have investigated the long term effects of HIV infection on DC populations [49, 50]. Dendritic cell numbers have been found to decrease with HIV infection. As DC numbers decline and HIV specific T cells undergo exhaustion, new methods of restoring immune function are necessary. The benefit of ex vivo DC is that they are unaffected by HIV replication and systemic immune activation present during HIV infection [51]. MoDC are generated outside the body, optimally matured, and loaded with HIV antigens before being returned to the patient, allowing them to bypass HIV-mediated immune dysfunction. Therefore, DC-based therapeutic vaccines can be used to stimulate a robust anti-HIV T cell response while at the same time training the immune system to attack specific viral epitopes [52].
The long term goal of DC-based HIV therapeutic vaccines is to be used in combination with or even replace HAART [53].

There have been more than 9 published clinical trials on DC-based therapeutic vaccines for HIV [53-62]. These studies have shown that DC vaccines can increase breadth, polyfunctionality, and T cell proliferation in patients [53]. However, many of these trials show limited immunogenicity and only modest viral control [60, 61]. These findings highlight the need for further improvements in DC vaccine technology.

Antigen selection is an important issue for HIV dendritic cell therapy. In previous studies, mRNA transfection of consensus HIV antigens has been evaluated. This approach has been able to induce immune responses in patients [53]. Whole inactivated virus has also been used as an antigen [54, 61]. Due to the rapid mutation rate and variability of an individual’s viral sequences, groups have also tested PCR of patient viral sequences to generate HIV antigens specific for an individual [60]. These custom antigens can be transfected into DC as mRNA. Based on the high variability of HIV within the population, selection of appropriate antigen(s) is key to the success of HIV DC therapies. An additional issue for HIV vaccination is decoy antigens and immune escape mutations. It is believed that HIV has epitopes that are strongly immunogenic and can be mutated without effecting viral fitness, leading to immune evasion. These epitopes “distracts” the immune system from epitopes more crucial to viral fitness [63]. To address this issue, groups are constructing antigens made only from highly conserved regions of the HIV genome [63, 64]. When tested in macaques, this approach has been found to broaden the magnitude and breadth of the immune response, while avoiding decoy epitopes [64]. It is the hope that by targeting these conserved regions of the HIV
genome, HIV DC therapy will be more effective and more individuals will be able to control viral replication.

1.5 Dendritic Cell Migration and Importance for Immunotherapy

Upon maturation, dendritic cells downregulate their phagocytic activity and acquire the ability to migrate out of the peripheral tissue and to the lymph node (Fig. 1.1). This migration is mediated by upregulation of the chemokine receptors such as CCR7 and CXCR4 [65, 66]. These receptors allow DC to migrate in response to chemokines CCL19 and CCL21. Unlike tissue DC, immature MoDC do not migrate to lymph nodes, due to a lack of CCR7 expression. Therefore it is necessary to mature MoDC to induce migration to the lymphoid organs upon injection in the patient [19]. There are several different maturation protocols used in the clinic to mature MoDC for immunotherapy. The most commonly used is a cytokine cocktail referred to as Mimic [23]. As mentioned previously, Mimic is a combination of TNF-α, IL-1β, IL-6 and prostaglandin E2 (PGE2). Mimic is a very effective form of maturation which yields a MoDC capable of stimulating IFN-γ producing CD4+ and CD8+ T cells [20]. However, due to issues with PGE2, some groups are exploring variations on the original Mimic formula.

PGE2 is a bioactive lipid derived from the metabolism of arachidonic acid [67]. PGE2 has been found to act as an enhancer to the cytokines used in Mimic, furthering the maturation of MoDC. PGE2 can enhance the T cell stimulatory capacity of the matured DC [65]. However, Kalinski et al. have shown that MoDC matured with PGE2 can generate a Th2 biased T cell response [27, 68, 69]. Whether this Th2 bias is correct is
controversial. Luft et al. published similar findings but suggested that Mimic matured DC could also induce the secretion of Th1 type cytokines as well [67, 70].

MoDC are known to express PGE2 receptors EP2 and EP4 [70]. Importantly, when MoDC are matured with Mimic, MoDC can migrate in response to CCL19 and CCL21 [71]. PGE2 is a powerful inducer of this DC migration. PGE2 is known to support CCR7 expression and promote migration to the lymph node [65, 67, 70]. When MoDC are matured with cytokines, LPS, or CD40L in absence of PGE2, migratory potential is dramatically decreased. A number of protocols can lead to DC maturation; however, only maturation protocols that contain PGE2 have routinely been shown to induce migration [70]. Interestingly, Luft et al. found that mature DC with or without migratory capacity expressed comparable levels of CXCR4 and CCR7 [70]. Despite similar CCR7 and CXCR4 levels, only PGE2 matured DC could migrate. They found that PGE2 induced migration by the E2 and E4 receptors which induced cAMP mobilization. They went further to show that PGE2-induced migration is mediated by mobilized cAMP. By synthetically inducing cAMP mobilization with forskolin, DC displayed migratory potential similar to that of PGE2 treated DC. Although PGE2 is necessary for MoDC migration, when peripheral blood DC were evaluated, migration was not dependent on PGE2 [70].

Other approaches have been taken to induce lymph node migration by MoDC. Nair et al. generated MoDC, loaded them with antigen, and matured them in situ [72]. In their protocol, immature MoDC were injected into Imiquimod adjuvant pre-treated skin to allow the MoDC to mature in a microenvironment similar to that encountered by tissue-resident DC. It was their hypothesis that this type of maturation would avoid the
induction of a Th2 immune response generated by Mimic matured DC. Nair et al. found that by pretreating skin with Imiquimod cream, Adjuprime, or poly-arginine, immature DC injected into the site developed lymph node migratory potential. Migration rates were less than those of mature DC but greater than immature DC injected into untreated skin. However, Imiquimod matured DC were less effective at stimulating a lytic CTL response than mature DC. Although further work needs to be done to fully develop this technology, in situ maturation in adjuvant-treated skin is an interesting concept that can be combined with other DC maturation strategies.

Another approach to increase MoDC migration is the alteration of the cytokine maturation cocktail. Several groups have explored maturation without the use of PGE2, with limited success. For example, Mailliard et al. have reported that their α-Type-1 DC maturation protocol can induce migration in MoDC [73]. By maturing MoDC with TNF-α, IL-1β, Poly I:C, IFN-α, and IFN-γ, these α-Type-1 DC develop a modest migration potential. However, levels of migration failed to reach that of Mimic matured MoDC. Importantly, α-Type-1 DC appeared to be superior to Mimic-DC at IL-12p70 secretion upon CD40L stimulation. Mimic DC are known to have an impaired ability to secrete IL-12p70 post maturation [68, 69]. While the α-Type-1 DC protocol is promising, it should be noted that the use of IFN-α may not be compatible with mRNA based electroporation methods. IFN-α is known to upregulate RNases in DC that may interfere with mRNA electroporation of antigens [24]. In addition, when α-Type-1 DC maturation protocols were evaluated in our laboratory, DC migration was not observed.

Due to the important role of migration in ex vivo DC vaccines, further research needs to be performed to optimize maturation protocols to allow for functional migration
while still maintaining the ability to stimulate a robust Th1 response and IL-12p70 secretion. Currently, PGE2 appears to be the best reagent to induce DC migration, but, as described in the next section, PGE2 dysregulates Th1 and IL-12p70 responses.

### 1.6 Issues with Prostaglandin E2

In order for dendritic cell immunotherapy to be effective, it is essential that the DC maturation protocol generate a robust Th1 type immune response. Cellular based immune responses are critical for in the control of tumors or viral infection. Current DC therapy protocols in the clinic have shown limited clinical efficacy. One potential cause of this limited efficacy is weak or dysfunctional DC activation and maturation [3, 24]. Without appropriate activation, DC are unable to provide costimulation or cytokine-mediated T cell activation, two of the three signals necessary to induce a robust adaptive immune response [24, 74]. Improperly activated DC may also be tolerogenic as previously discussed [24].

The cytokine cocktail Mimic is a standard reagent to mature monocyte-derived DC for immunotherapy studies. While Mimic effectively matures and activates DC, the addition of PGE2, required for migration of DC to the lymph node [65, 66], has been found to have unintended side effects. For example, PGE2 has been shown to be a potent inducer of IL-10. IL-10 is an anti-inflammatory cytokine known to inhibit dendritic cell function. Maturation of dendritic cells with PGE2 leads to a high IL-10/IL-12p70 ratio (Fig. 1.3) [67, 69]. IL-10 is known to suppress the Th1 response [75]. Also, IL-10 has the ability to inhibit IL-12p70 production from dendritic cells. Harizi et al. have very elegantly shown that the principal mechanism of PGE2 inhibition of DC and IL-12p70
secretion was IL-10 mediated [76]. When IL-10 was blocked by an anti-IL-10 antibody, dendritic cell function and IL-12p70 secretion were restored [76].

IL-10 also has a profound effect on the type of immune response a DC generates. DC treated with IL-10 were unable to induce a Th1 response. IL-10 pretreated DC have also been shown to develop tolerance and induce T cell anergy [77]. Similarly, studies have shown that PGE2 leads to Th2 polarization of the immune response [27, 76]. It is possible that a Th2 based immune response could be beneficial for an anti-tumor immune response, however a Th1 response would likely be preferable.

![Figure 1.3: MoDC IL-10 and IL-12p70 secretion in response to PGE2](harizi_h_j_immunol_2002_mar_1_168(5):2255-63)

PGE2 can also lead to dendritic cell exhaustion upon restimulation. Following restimulation with CD40L or LPS, PGE2 matured DC are unable to secrete IL-12p70 [67-69, 78, 79]. Langerkamp et al. showed very nicely that when DC are exposed to PGE2, secretion of TNF-α and IL-12p70 is drastically impaired upon CD40L stimulation (Fig. 1.4) [79]. This exhaustion is a critical concern for DC vaccine protocols.
Following maturation with Mimic, DC that migrate to the lymph node will be unable to respond to CD40L based stimulation from T cells. This exhaustion is expected to lead to a Th2 biased, unsynchronized immune response, and to induce tolerance to the presented antigen [23]. Together these findings highlight the importance of finding an alternative to PGE2 for DC immunotherapy. It also raises the question of whether the use of PGE2 is masking the clinical efficacy of DC immunotherapy trials. Further research is necessary to develop a maturation protocol more suitable to DC therapeutic vaccines that avoids the use of PGE2.

Figure 1.4. Cytokine-production of MoDC post PGE2 maturation.
DCs were stimulated with LPS for 8, 24 or 48 h (DC-8, hatched bars; DC-24, shaded bars; DC-48, open bars), washed and recultured in the absence or in the presence of CD40L. Cytokine production was measured in the 24-h culture supernatant. Cytokine amounts in the 24-h culture supernatant of LPS-stimulated immature DCs are shown for comparison (filled bars). DCs that had been primed for 8 h with LPS, washed and recultured in medium were found to produce higher amounts of IL-12 than did LPS-stimulated immature DCs (1.4- to 8-fold, mean 3.7-fold, in six DC preparations from different donors).

1.7 Overview of Latent Membrane Protein-1

Epstein-Barr Virus (EBV) is a γ-herpesvirus that causes lymphoproliferative diseases and infectious mononucleosis [80]. It is believed to infect more than 90% of individuals globally. EBV is associated with several human malignancies including Hodgkin’s lymphoma, Burkitt’s lymphoma, and nasopharyngeal carcinoma [80, 81]. It was discovered that a critical EBV protein involved in cell transformation is Latent Membrane Protein-1 (LMP1) [82, 83]. LMP1 is the principal oncoprotein of EBV and has been found to be necessary for transformation in these and other human malignancies [84]. LMP1 expression is frequently observed within EBV derived tumors.

LMP1 contains a short 24 amino acid N-terminal cytoplasmic tail followed by a six membrane-spanning domain (Fig. 1.5). The C-terminal end of LMP1 contains a 200 amino acid cytoplasmic tail known to be responsible for LMP1 mediated intracellular signaling [80]. The membrane-spanning domain of LMP1 is also critical for its function. This domain is responsible for ligand-independent oligomerization, leading to oligomerization-dependent signaling via the C-terminal cytoplasmic tail [84]. Interestingly, LMP1 has evolved as a viral mimic of the human CD40 receptor, with only modest differences in the TRAF mediated signaling between these two proteins [83, 85, 86]. However, unlike the ligand dependent signaling of CD40, LMP1 signaling is constitutively active. The LMP1 membrane spanning domain is responsible for this ligand independent oligomerization and resulting constitutive activity. The LMP1 membrane spanning domain is also important in trafficking the protein to lipid rafts on the cell membrane where it oligomerizes and interacts with cell signaling molecules [83].
Again, the C-terminal tail of LMP1 is responsible for intracellular signaling (Fig. 1.5). LMP1 signaling is known to constitutively activate NF-κB, MAP kinase, IRF7, and PI3 kinase pathways [84]. There are 3 signaling regions of the C-terminal tail, termed C-Terminal Activating Regions 1-3 (CTAR1-3) [80, 84, 87]. Each CTAR serves a different signaling function. For instance, CTAR1 activates the NF-κB non-canonical pathway through the recruitment of TRAF 1, 2, 3, and 5, and CTAR2 activates the canonical NF-κB pathway through the recruitment of RIPP and TRADD (Fig. 1.5) [82, 88]. LMP1 also mediates IRF7 signaling. IRF7 was found to strongly interact with the TES2 region of the LMP1 C-terminal tail [84]. LMP1 activation of the JNK and p38 pathways is less clear cut. Although JNK and p38 activation is believed to be CTAR2 dependent, it may also have some CTAR1 involvement [83]. Due to the complexity of LMP1 signaling, not all signaling pathways are completely understood.

Importantly, LMP1 signaling is known to induce expression of genes important for cell survival and blockage of apoptosis, upregulation of cytokines and cytokine receptors, and cell migration [62]. This pro-survival anti-apoptotic phenotype is likely involved in LMP1-mediated transformation. However, because LMP1 signaling can also induce the activation of B-cells [81, 85], we proposed to repurpose LMP1 for use as a molecular adjuvant to activate dendritic cells. We believe that LMP1 mediated signaling, which is similar to that of CD40, can enhance DC maturation and survival. Gene expression data has also shown that IFN based maturation is more effective than Mimic
maturation at inducing a strong Th1 immune response [21]. Due to the strong induction of IRF7 by LMP1 [84], it is possible that LMP1 transduced DC may be superior to Mimic matured DC at stimulating a robust Th1 immune response.
**Chapter 2-Materials and Methods**

**Cloning of LMP-GFP Fusion Protein**

LMP1 was fused to green fluorescent protein (GFP) to track the protein expression and location of LMP1 within a cell. Constructs were cloned such that GFP was fused to C-terminal cytoplasmic tail of full-length LMP1. The fusion protein was then cloned into a pcDNA3.1 expression vector. Western blot analysis and flow cytometry were used to confirm proper protein expression in transfected 293T cells.

For microscopy, AD293 cells were plated in glass bottom culture dishes (Greiner Bio-One) and transfected with either pcDNA-GFP or pcDNA-LMP1-GFP using Lipofectamine 2000 (Invitrogen). After 36 hours, cells were imaged using a Leica SP5 confocal microscope.

**Luciferase reporter assay**

NF-κB and IFN-β induction was measured using a dual-luciferase reporter assay system (Promega) according to manufacturer’s instructions. 293T cells seeded on 24-well plates were transiently transfected with 30 ng of either the NF-κB or IFN-β firefly luciferase reporter plasmid together with 4ng pRL-TK and 300 ng of various expression plasmids or empty control plasmids. As positive controls, pcDNA3.1-FLAG-TRAF6 and pcDNA3.1-ΔRIG-I were used. 36-48h following transfection, luciferase activity was measured on the total cell lysate.
**PBMC Samples**

All samples were collected in sodium Heparin unless otherwise noted. For HIV infected patient samples, patients were recruited from the University of Miami AIDS Clinical Research Unit. A total of 4 patient samples were evaluated. All patients signed informed consent in accordance with the University of Miami Institutional Review Board. Uninfected human blood samples (buffy coat) were purchased from Continental Services Group Inc., Miami, FL and were negative for HIV, HBV, and HCV infection.

**Monocyte-Derived Dendritic Cell Preparation**

Whole blood from buffy coat or from HIV+ patients was centrifuged over ficoll (GE Healthcare) at 1000xg for 25 min at room temperature. The monocyte layer was re-suspended in PBS, centrifuged sequentially at 1800 rpm for 10min, 1100rpm for 10min, then 1000rpm for 10 min with aspiration and resuspension between each spin. PBMC were finally resuspended in complete media: RPMI1640 (Hyclone) containing 5% human AB serum Male only (Lonza), PenStrep (1x), and L-glutamine. To isolate monocytes, $8 \times 10^7$ HIV PBMC were placed in a T75 flask or $2 \times 10^8$ buffy coat PBMC in a T175 flask in complete media and cultured for 2 hours at 37°C in 5% CO2. Unattached cells were removed by washing with warm media three times. Unattached cells (defined as peripheral blood lymphocytes, PBL) were pelleted, resuspended in 80% FBS + 10% Glucose + 10% DMSO, and cryopreserved. The adherent monocyte cells were cultured in 20 ml (T75) or 40 ml (T175) of media containing 500U/ml IL-4.
(R&D systems) and 1000U/ml GM-CSF (BERLEX Inc). After 5 days of culture, flasks containing immature DC were placed at 4°C for 30 minutes to detach DC, then collected by gentle pipetting to isolate unattached and loosely attached cells. Any remaining adherent cells were discarded.

**mRNA Preparation and DC Transfection**

LMP1, GFP, Gag, and human gp100 were each cloned into the expression vector pGEM4Z/A64. The mMachine mMessage RNA in vitro transcription kit (Ambion) was used to generate mRNA from all constructs. GFP mRNA was used as a control in all experiments. Day 5 DC were washed, resuspended in OPTI-MEM medium (GIBCO) and transfected with 10µg mRNA. A total of \(1 \times 10^6\) immature DC in 100µl OPTI-MEM were placed in a 0.4µm GenePulser cuvette (Bio-Rad) for each electroporation. Electroporation was performed on a GenePulser Xcell (Rio-Rad) with exponential decay at 300V and 150µF. 350V was also used were indicated. The transfected DC were transferred to 6-well plates with 3ml complete media containing Mimic cytokine mix (5 ng/ml TNF-\(\alpha\) (R&D), 5ng/ml IL-1\(\beta\) (R&D), 750ng/ml IL-6 (R&D)) + 1µg/ml PGE2 (Sigma)). Cells were cultured overnight at 37°C in 5% CO\(_2\). GFP mRNA transfection efficiency was confirmed by flow cytometry.
MoDC Maturation and Activation Post LMP1 Electroporation

On Day 5 DC were harvested and electroporated as described above. Mimic cytokine cocktail was used as a positive control (5 ng/ml TNF-α (R&D), 5ng/ml IL-1β (R&D), 750ng/ml IL-6 (R&D)) + 1μg/ml PGE2 (Sigma)). After 36 hours, cultures were placed at 4°C for 30 minutes to detach DC, then collected by gentle pipetting to isolate unattached and loosely attached cells. DC were stained for markers of maturation and activation and run on an LSRFortessa (anti-human CD14 clone M5E2, CD86 clone 2331 (FUN-1), CD80 clone L307.4, HLA-DR clone TU36, CD83 clone HB15e, CD40 clone 5C3, CD197 clone 3D12, and CD11c clone 3.9) (BD Bioscience).

Human and Mouse CBA

Mouse BMDC (see below for protocol) and human MoDC culture supernatants were collected every 12 hours after electroporation or transduction. Supernatants were analyzed using the Mouse Inflammatory Cytokine CBA kit or the Human Inflammatory Cytokine CBA kit (BD Bioscience) according to manufacturer’s instructions and run on an LSRFortessa. Cytokine values were calculated and represented as pg/ml.

In vitro Migration Assay (Electroporation)

Day 5 DC were harvested and electroporated as described above. DC were either electroporated with 10μg GFP or LMP1 mRNA. Mimic cytokine cocktail was used as a positive control. DC were then cultured for 48 hours in complete DC
media. To harvest, cultures were placed at 4°C for 30 minutes to detach DC, then collected by gentle pipetting to isolate unattached and loosely attached cells. 1.5x10^5 mature dendritic cells in 150 µL were added to 8 µm transwells (Greiner Bio-one) in triplicate. Transwells were held in a 24 well plate with 600 µL of human dendritic cell media containing 150 ng/ml of CCL19 (Peprotech). After a 90 minute incubation at 37 °C, transwells were removed and dendritic cells in the lower chamber removed with EDTA and counted.

**Tumor DC Therapy Flank (Electroporation)**

C57BL/6 mice (7–8 week old) were injected intradermally in the flank with 5.0x10^4 B16-F10 melanoma cells. After 3 days, mice were vaccinated with 1x10^6 DC intradermally every 7 days for a total of 3 vaccinations. BMDC (see method below) were either electroporated with mRNA of gp100 and GFP, gp100 and LMP1, or gp100 and GFP matured with Mimic cytokine cocktail. Electroporation was performed as outlined above with 10µg of each mRNA. As a positive control, GM-CSF expressing B16/F10 tumor cells (GVAX), kindly provided by Dr. Eli Gilboa, were irradiated (5,000 rad) and 1 x 10^6 cells were injected subcutaneously every 3 days for a total of 3 vaccinations. Tumor area was measured every 2 days. Mice were euthanized when tumors reached 15x15mm.

**IL-12p70 secretion assay from human MoDC.**

Untreated DC were stimulated with LPS (250ng/ml) and IFN-γ (1000u/ml) or electroporated at either 300V or 350V. After a 24 hour recovery period, DC were
stimulated with LPS and IFN-γ. Supernatants were collected every 12 hours. Immature DC were also transduced with Ad5-GFP at an MOI of 1,000. Post-transduction, DC were stimulated with LPS+IFN-γ. Supernatants were collected at 16 and 36 hours. All cytokine analysis was done by Cytometric bead array using the human IL-12p70 CBA flex set (BD Bioscience).

**Survival assay**

Human MoDC were prepared as described above. On day 5 DC were harvested and electroporated with 10μg GFP mRNA. DC were also transduced with Ad5-GFP at an MOI of 100. $5 \times 10^5$ DC were plated in complete DC media in a 12 well plate in triplicate in such a way that 3 wells would be harvested for each treatment daily for 5 days. Every 24 hours DC were harvested, counted, and the viability was assessed using Trypan Blue. DC survival was determined by comparing the initial amount of DC plated to the recovered amount.

**Pmel Assay (Route of Transfection)**

Pmel-1 transgenic (Thy1.1+Vβ13+) mice were bred in house. BMDC were generated from C57BL/6 mice as described below. On day 4, DC were transduced at an MOI of 50 with Ad5-gp100 or electroporated with 10μg gp100 mRNA. Mimic maturation cocktail was used to mature DC where indicated. On day 5, $1 \times 10^6$ DC were injected intradermally into the flank of C57BL/6 mice that had been adoptively transferred with $1 \times 10^6$ purified CD8+ T cell isolated from a Pmel-1 mouse 24 hours previously. As a positive control, LPS (Sigma Aldrich)
mixed with gp100 peptide (KVPRNQDWL) (American Peptide) was injected subcutaneously. After 5 days, spleens were harvested and processed for single cell suspensions. Cells were stained with anti-mouse CD3e clone 500A2, anti-mouse CD8a PerCP clone 53-6.7, anti-mouse Thy-1.1 clone OX-7 (BD Bioscience). Cells were run on an LSRFortessa (BD Bioscience) and data represented as percent of CD3+ CD8+ T cells that were Thy1.1+.

**Production of Recombinant Adenovirus**

Replication deficient adenovirus (pAdEasy-1) containing codon-optimized TPA-Gag, human gp100, or GFP (as an irrelevant antigen control), were generated as described by the manufacturer’s instructions (AdEasy Adenoviral vector system, Agilent tech). The genes were PCR amplified and cloned into the pAdenoVator-CMV5 shuttle vector (Qbiogene) and sequenced to confirm their correct sequence. The CMV5-shuttle vectors were then electroporated into BJ5183 cells containing the pAdEasy-1 plasmid to induce homologous recombination. The recombined pAdEasay-1 vectors were then linearized and transfected into AD293 cells (Stratagene).

Adenovirus expressing LMP1 was constructed using the Adeno-X Tet-On 3G inducible system (Clontech). LMP1 sequence was cloned together with an IRES (internal ribosomal entry site) and GFP gene to allow LMP1 protein expression to be tracked by GFP fluorescence. LMP1-IRES-GFP was then cloned into the Adeno-X system as described by the manufacturer’s instructions. Following PCR of the complete gene, LMP1-IRES-GFP was cloned into the
linearized Ad5-Tet-On genome using the InPhusion cloning kit (Clontech).
Ligated plasmid was grown in Stellar competent cells. Following sequencing to
confirm the correct gene sequence, viral vectors were linearized and transfected
into AD293 cells (Stratagene).

Recombinant virus was propagated in AD293 cells, then purified and
concentrated using the Adeno-X Mega purification kit (Clontech). To determine
infectious units (CFU), viruses were titered using the Adeno-X Rapid titer kit
(Clontech).

**Western Blot to Confirm Protein Production:**

1x10⁶ 293T cells were transduced with 1x10⁶ CFU of each viral construct. After
48 hours, cells were harvested and lysed in RIPA buffer (Biorad) for Western blot
analysis. Western blot was performed as previously described [89]. Proteins were
denatured with 2% SDS and 1% DTT before they were loading on a 4-15%
gradient Tris Glycine-SDS poly-acrylamide gels (Bio-Rad), electrophoresed, and
blotted onto PVDF membrane (Pierce). The membrane was blocked using 5%
(w/v) dry milk and then probed with Mouse anti-EBV LMP-1 antibody (Santa
Cruz Biotechnology), followed by incubation with Peroxidase-Conjugated
Donkey Anti-mouse IgG (Jackson Immunoresearch). The protein band was
developed onto X-ray film using ECL detection reagent (Amersham).
**RT-PCR analysis of LMP1 mRNA**

For the measurement of LMP1 mRNA levels in transduced AD293 cells, quantitative RT-PCR was performed. $1 \times 10^6$ AD293 cells were transduced with $1 \times 10^6$ CFU Ad5-LMP1 with or without $1 \mu$g/ml doxycycline added to culture media. pcDNA3.1-LMP1 plasmid was transfected as a positive control using Lipofectamine 2000 (Invitrogen) according to manufacturer protocol. After 48, total RNA was prepared using the RNeasy kit (Qiagen), and reverse transcribed in a 20 µl reaction containing 0.1 µg of total RNA, 0.1 µg of oligo(dT), 200 units of reverse transcriptase (Finnzymes) and 0.2 µM each of dATP, dCTP, dGTP and dTTP. After 1 hr incubation at 40°C, cDNA products were generated. Real-time PCR then was performed as previously described [90] using the Power SYBR Green Supermix (Applied Biosystems) with primers specific to LMP1. For normalization, GAPDH and β-actin real-time PCR was carried out on the same samples. Normalized mRNA levels for each transcript were calculated as $(1/2\Delta Ct \times 1,000)$, where $\Delta Ct$ value = $Ct$ (test mRNA) - $Ct$ (GAPDH mRNA). To control for contamination with genomic DNA, parallel amplifications were performed in the absence of reverse transcriptase. These were uniformly negative.

**SEAP Assay**

A NF-κB reporter cell line (293-SEAP) was used to monitor NF-κB activation by LMP1. This 293-derived cell line contains the gene for secreted embryonic alkaline phosphatase (SEAP) under the control of an NF-κB promoter [91]. 8.0x10⁴ 293-SEAP reporter cells, grown in DMEM medium with 10% FBS, were
plated in each well of a 96-well plate. Viral stocks of Ad5-LMP1 were serially
diluted and added to the reporter cells in triplicate, in the presence or absence of
1µg/ml doxycycline (Alfa Aesar). After 36 h, 10 µl/well of the supernatants was
added to the wells of a 96-well assay plate together with 100 µl/well of QUANTI-
Blue Alkaline Phosphatase substrate (InvivoGen). The plates were incubated for
20 min at 20°C and OD was read at 650 nm.

Mice
Female C57BL/6 mice (7–8 week old) were used in all experiments except for
vaccinia challenges in which female BALB/c mice (7–8 week old) were used.
Animals were housed at the University of Miami under the guidelines of the
National Institutes of Health (NIH, Bethesda, MD). All animal experiments were
performed in accordance with national and institutional guidance for animal care
and were approved by the IACUC of the University of Miami.

Generation of BMDC
Bone marrow-derived murine DC (BMDC) were generated by standard methods
[92] with the following modifications: Bone marrow cells were obtained from
C57BL/6 mice and washed in RPMI 1640 media. The cells were then placed in
tissue culture treated T75 flasks at a concentration of 1 x 10^6 cells per ml in 20 ml
complete RPMI (RPMI 1640 with 10% FBS, 20 µg/ml gentamycin sulfate, 50 µM
2-mercaptoethanol), and 20 ng/ml murine recombinant GM-CSF and 10 ng/ml
murine recombinant IL-4 (Peprotech)). Cells were cultured at 37°C, 5% CO2 and
on day 3, media was replaced with fresh complete RPMI containing cytokines. On day 5, DC were harvested by incubating at 4°C for 30 minutes to detach DC, then collected by gentle pipetting to isolate unattached and loosely attached cells. DC were then washed and resuspended in complete RPMI at 5 x 10^5 cells/ml.

**BMDC Maturation and Activation**

BMDC were generated from C57BL/6 mice as described above. 1x10^6 DC were plated in each well of 6-well tissue culture treated plates in a volume of 800ul. DC were transduced with Ad5-LMP1 or Ad5-GFP control at an MOI of 50. DC were incubated with virus at 4°C for 1 h, followed by 3 h at 37°C. Complete media was then added to 3ml. As a positive control, Mimic cytokine cocktail adapted for mice was used to mature DC (5 ng/ml TNF-α (Peprotech), 5ng/ml IL-1β (Peprotech), 750ng/ml IL-6, 1µg/ml PGE2 (Sigma)). Cells were incubated for 36 hours at 37°C, harvested, and stained with the following antibodies: anti-mouse CD80 clone 16-10A1, anti-mouse CD86 clone GL1, anti-mouse CD40 clone 1C10, anti-mouse CD83 clone Michel-17, anti-mouse MHC Class II (I-A/I-E) clone M5-114.15.2, and anti-mouse CCR7 clone 4B12 (all from eBioscience). All tubes were also stained with hamster anti-mouse CD11c clone N418 PE-Cyanine7 conjugate (eBioscience) to allow for gating on CD11c+ DC. After flow cytometry analysis, the mean fluorescence intensity (MFI) for each antibody was calculated for CD11c+ dendritic cells under each experimental condition. Three independent wells were analyzed for each condition.
In Vivo Migration

BMDC were generated from C57BL/6 mice as described above. BMDC were transduced at an MOI of 50 with Ad5-LMP1, Ad5-Gag negative control, or Ad5-Gag DC matured with Mimic cytokine cocktail. After 36 hours, DC were CFSE labeled and injected intradermally into the right flank of C57BL/6 mice. After 48 hours, the inguinal lymph nodes were dissected and processed for single cell suspensions. Cells were stained with hamster anti-mouse CD11c clone N418 PE-Cyanine7 conjugate (eBioscience) and run on an LSRForstessa. The total number of migrated CFSE+ CD11c+ dendritic cells was calculated based upon volume.

Ad5-LMP1 Pmel Assay

Pmel-1 transgenic (Thy1.1+Vβ13+) mice were bred in house. BMDC were generated from C57BL/6 mice as described above. On day 3, DC were transduced at an MOI of 50 with Ad5-gp100. On day 4, DC were with transduced at an MOI of 50 with Ad5-GFP, Ad5-GFP + Mimic maturation cocktail, or Ad5-LMP1 and cultured in complete media containing Doxycycline (1μg/ml). On day 5, 1x10^6 DC were injected intradermally into the flank of C57BL/6 mice that had been adoptively transferred with 1x10^6 purified CD8+ T cell isolated from a Pmel-1 mouse 24 hours previously. For a positive control, LPS (Sigma Aldrich) + gp100 peptide (KVPRNQDWL) (American Peptide) were injected subcutaneously. After 5 days, spleens were harvested and processed for single cell suspensions. Spleen preps were stained with anti-mouse CD3e clone 500A2,
anti-mouse CD8a PerCP clone 53-6.7, anti-mouse Thy-1.1 clone OX-7(BD Bioscience). Cells were run on an LSRFortessa (BD Bioscience) and data represented as percent of CD3+ CD8+ T cells that were Thy1.1+.

**Tumor DC Therapy Flank**

C57BL/6 mice (7–8 week old) were injected intradermally in the flank with 5.0x10^4 B16-F10 melanoma cells. After 3 days, mice were vaccinated with 1x10^6 DC intradermally every 3 days for a total of 6 vaccinations. DC were either transduced with Ad5-gp100 and Ad5-GFP, Ad5-gp100 and Ad5-LMP1, or Ad5-gp100 and Ad5-GFP matured with Mimic cytokine cocktail. As a positive control, GVAX was irradiated (5,000 rad) and 1 × 10^6 cells were injected subcutaneously at the same vaccination schedule as DC. Doxycycline (2mg/ml) was added to the mouse water bottles to insure continued expression of the Tet-inducible LMP1 protein. Tumor area was measured every 2 days. Mice were euthanized when tumors reached 15x15mm.

**Tumor DC Therapy Lung**

C57BL/6 mice (7–8 week old) were injected intravenously with 5.0x10^4 B16-F10 melanoma cells. After 3 days, mice were vaccinated with 1x10^6 DC intradermally every 3 days for a total of 6 vaccinations. DC were either transduced with Ad5-gp100 and Ad5-GFP, Ad5-gp100 and Ad5-LMP1, or Ad5-gp100 and Ad5-GFP matured with Mimic cytokine cocktail. As a positive control, GVAX, were irradiated (5,000 rad) and 1 × 10^6 cells were injected subcutaneously at the same
vaccination schedule as DC. Doxycycline (2mg/ml) was added to the mouse water bottles to insure continued expression of the Tet-inducible Ad5-LMP1. 21 days after tumor injection, mice were sacrificed and lungs were harvested.

**Vaccinia Viral challenge**

7-8 week-old female Balb/C mice were immunized (five mice per group) with $1.0 \times 10^5$ dendritic cells transduced with Ad5-Gag, Ad5-Gag and Ad5-LMP1, or Ad5-Gag and matured with Mimic cytokine cocktail. Vaccinia-Gag challenge assay was performed as previously described [93]. One month following immunization, mice were challenged i.p. with $1 \times 10^7$ viral particles vaccinia-Gag virus. Five days after challenge, mice were sacrificed and both ovaries and fallopian tubes were removed and homogenized in 500ul PBS. For measurement of virus titers, samples were sonicated, and evaluated in triplicate by 10-fold serial dilution on CV-1 cells plated in 24 well plates. After 48 hour incubation, plates were stained with 0.1% (w/v) crystal violet in 20% ethanol. Plaques were counted to determine PFU of virus. Data was presented as PFU/mouse which was calculated based upon the recovered volume of homogenate.

**MoDC Maturation and Activation**

On Day 5 DC were removed from their flasks as described above and $1.0 \times 10^6$ DC were plated in a 6 well plate (800ul). DC were transduced with Ad5-Gag or Ad5-LMP1 at an MOI of 100. DC were first cultured in the fridge for 1 hour, then incubated at 37°C for 3 hours. 2 ml fresh DC media was then added for overnight
culture. Following 36 hours of culture in the presence of Doxycycline (1\(\mu\)g/ml) or Mimic cytokine cocktail, DC were placed at 4°C for 30 minutes to detach DC, then collected by gentle pipetting to isolate unattached and loosely attached cells. DC were stained for markers of maturation and activation as described above.

**MoDC Cytokine Secretion and Restimulation Assay**

On Day 5 DC were removed from their flasks as described above and 1.0x10^6 DC were plated in a 6 well plate (800ul). DC were transduced with Ad5-GFP or Ad5-LMP1 at an MOI of 100. DC were first cultured in the fridge for 1 hour, then incubated at 37°C for 3 hours. 2 ml fresh DC media was then added for overnight culture. Other DC were matured with either Mimic cytokine cocktail or LPS (250ng/ml) (Sigma) plus IFN-\(\gamma\) (1000u/ml) (R&D Systems). Cytokines were collected every 12 hours for 48 hours at which point cells were washed and restimulated with LPS (250ng/ml) plus IFN-\(\gamma\) (1000u/ml). After 24 hours supernatants were collected and all samples were assayed using the Human Inflammatory Cytokine CBA kit (BD Bioscience).

**DC Maturation Protocol Test for In vitro Migration**

Day 5 immature human MoDC were matured using various protocols from the literature. DC were matured with the indicated combinations of Mimic, LPS (250ng/ml), or Mega-CD40L (100ng/ml) (Enzo Life Sciences). Each protocol was tested with and without PGE2 to determine the level of migration. After 48 hours maturation, DC were placed at 4°C for 30 minutes to detach DC, then
collected by gentle pipetting to isolate unattached and loosely attached cells. 1.5x10^5 mature dendritic cells in 150 µL were added to 8 µm transwells (Greiner Bio-one) in triplicate. Transwells were held in a 24 well plate with 600 µL of human dendritic cell media containing 150 ng/ml of CCL19 (Peprotech). After 90 minutes incubation at 37 °C, transwells were removed and all dendritic cells in the lower chamber removed with EDTA and counted.

**In vitro Migration Assay (Ad5-LMP1)**

Day 5 DC were removed from their flasks as described above and 1x10^6 DC were plated in a 6 well plate (800ul). DC were transduced with Ad5-GFP or Ad5-LMP1 at an MOI of 100 or 500. To transduce DC, cells were first cultured in the fridge for 1 hour, then incubated at 37°C for 3 hours. 2 ml fresh DC media was then added for overnight culture. Following 36 hours of culture in the presence of Doxycycline (1µg/ml) or Mimic cytokine cocktail, a migration assay was carried out as described above.

**Allogeneic Mixed Leukocyte Reaction (MLR)**

On Day 5, DC were removed from their flasks as described above. 1.0x10^6 DC were plated in a 6 well plate (800ul). DC were transduced with Ad5-GFP or Ad5-LMP1 at an MOI of 100. To transduce, DC were first cultured in the fridge for 1 hour, then incubated at 37°C for 3 hours. 2 ml fresh DC media was then added for overnight culture. Following 36 hours of culture in the presence of Doxycycline (1µg/ml) or Mimic cytokine cocktail, plates were placed at 4°C for
30 minutes to detach DC, then collected by gentle pipetting to isolate unattached and loosely attached cells. MLR was performed as previously described [94]. 1.0x10^4 DC were cultured with 1.0x10^5 CFSE labeled allogeneic PBMC for 7 days. Cells were harvested and stained with anti-human CD3 clone SK7, anti-human CD8 clone RPA-T8, and anti-human CD4 clone RPA-T4 (BD Bioscience) before being run on an LSRFortessa. Proliferation of T cells was calculated in comparison to an unstimulated control.

Enzyme Linked Immunospot (ELISPOT) Assay

On Day 5 immature DC were removed from their flasks as described above and 1.0x10^6 DC were plated in a 6 well plate. DC were transduced with Ad5-GFP (negative control) or Ad5-Gag at an MOI of 100 as described above. DC were cultured in a total of 3 ml fresh DC media for overnight culture. On day 6, DC were transduced with Ad5-LMP1 following the same procedure as for Ad5-Gag.

Following 36 hours of culture in the presence of Doxycycline or Mimic cytokine cocktail, DC were placed at 4°C for 30 minutes to detach DC, then collected by gentle pipetting to isolate unattached and loosely attached cells. Donor autologous PBL (peripheral blood lymphocytes) that were cryopreserved (90% FBS, 10% DMSO) on day 0 after monocyte isolation were thawed and cocultured at DC:PBL ratio of 20,000:100,000 cells for 12 days. After coculture, cells were harvested and counted. IFN-g and IL-2 ELISPOT assays were performed to determine antigen specific cytokine secretion induced by the DC:T cell coculture. ELISPOT assays were carried out per the manufacturer’s
instructions (R&D Systems) using 96-well MAIP plates (Millipore). A total of 1×10^5 cells were added to each well of the plate, and stimulated for 18 h at 37°C, 5% CO2, in the presence of HIV-1 Gag peptide pool (5 μg/ml or as described). An OVA peptide (negative control) and PMA/Ionomycin (positive control) were also included. After 18h, spots were developed with AEC substrate kit (BD Bioscience), according to manufacturer’s instructions. The membrane was read by automated reader (CTL Immunospot) for quantitative analyses of the number of IFN-γ or IL-2 spots forming counts (SFC) per million cells plated, subtracting negative control values.

**Flow Cytometry Analysis**
Flow cytometric data was analyzed using FlowJo 7.6.4.

**Statistical Analysis**
Graph pad Prism 6.0 software was used to calculate significance using a one-way ANOVA, followed by either a two-tailed Student's t test or a Mann-Whitney test as noted. A log-rank test was used to determine the significance of differences between groups in Kaplan-Meier survival plots. A p value of 0.05 was considered significant. In all figures, p values are labeled by asterisks denoting p < 0.05 (*), p < 0.01 (**), and p < 0.001 (***)
Statement of Purpose

A major goal of our laboratory is to enhance dendritic cell immunotherapy for the treatment of cancer and HIV. Many studies have been conducted in attempts to increase the efficacy of DC immunotherapy through the optimization of DC maturation protocols. However, there are still critical issues in the field when it comes to clinical efficacy. We have evaluated the benefit of using novel molecular adjuvants to mature dendritic cells and improve DC vaccination.

The objective of this project was to assess the benefits of using latent membrane protein-1 (LMP1) to enhance dendritic cell immunotherapy. To determine these benefits, LMP1 was cloned into a replication incompetent Ad5 vector for transduction of DC. We first investigated the in vitro effects of LMP1 on DC maturation, migration, and cytokine production in both human and mouse DC. Second, we assessed the in vivo benefits of DC immunotherapy using LMP1 transfected DC in tumor models, vaccinia-Gag prophylactic vaccination, and DC:T cell cocultures of human patient samples.
Chapter 3-Results

3.1. Evaluating the effects of LMP1 mRNA electroporation on human monocyte derived dendritic cells

3.1.1. Confirming oligomerization of LMP1 using an LMP1-GFP fusion protein.

It is thought that the mechanism by which LMP1 signals and activates cells is through ligand-independent oligomerization in the cell membrane mediated by the six membrane-spanning domains of LMP1. This spontaneous oligomerization enables the intracellular tail of LMP1 to interact with cellular proteins and mediate ligand independent signaling [80, 82, 84, 95]. While the oligomerization of LMP1 has been previously established by biochemical methods, it was decided to confirm oligomerization of our LMP1 construct. To determine whether oligomerization was occurring, we constructed a fusion protein where green fluorescent protein (GFP) was fused to the C-terminal cytoplasmic domain of full-length LMP1. This fusion protein allowed for the visualization of LMP1 oligomerization by fluorescent microscopy.

When control plasmid pcDNA-GFP was transfected into 293T cells, a diffuse green fluorescent signature was observed within the cell (Fig. 3.1A), presumably due to GFP molecules evenly dispersed within the cytosol. However, when LMP1-GFP was transfected into 293T cells, punctate fluorescence was observed (Fig. 3.1B). This punctate GFP was likely caused by oligomerization of LMP1-GFP on the cell membrane. If our LMP1 construct did not oligomerization, a diffuse GFP pattern would be expected, as was observed for pcDNA-GFP.
Figure 3.1: Expression of LMP-GFP fusion protein. AD293 cells were transfected with an expression vector containing either GFP or LMP-GFP fusion protein. After 36 hours cells were imaged. Light image and GFP fluorescence image were merged.

Figure 3.2: Biological Activity of Ad5-LMP1. 293T cells were transfected with either NF-κB (A) or IFN-β (B) firefly luciferase reporter plasmid together with pRL-TK and either LMP1 or control expression plasmids. As positive controls, pcDNA3.1-FLAG-TRAF6 and pcDNA3.1-ΔRIG-I were used. 36-48h later, luciferase activity was measured in total cell lysate.
3.1.2 LMP1 induced NF-κB and IFN-β expression

To test the signaling activity of our LMP1 construct, pcDNA3.1-LMP1 was transfected into 293T cells together with NF-κB or IFN-β luciferase reporter plasmids. Flag-TRAF6 and Δ-RIGI vectors were used respectively as NF-κB and IFN-β positive controls. LMP1 induced NF-κB levels >100-fold higher than the GFP control (Fig. 3.2A). LMP1 induced IFN-β levels >500-fold higher than the GFP control (Fig. 3.2B). These data suggest that our LMP1 construct is biologically active and providing the expected intracellular signaling.

3.1.3. Dendritic cell maturation and activation

Based on the signaling observed in the NF-κB and IFN-β luciferase reporter assays, we decided to test the effect of LMP1 on dendritic cell maturation and activation. LMP1 in vitro transcribed mRNA was electroporated into human monocyte-derived dendritic cells (MoDC). Mimic cytokine cocktail was used as a positive control. Transfection of LMP1 enhanced maturation and activation of MoDC compared to a Gag transfection control (Fig. 3.3A). Increased levels of CD80, CD83, CD40 and CCR7 were observed. Levels of CD40 expression were higher than the Mimic matured positive control.

Next, we evaluated the effect of LMP1 transfection on DC proinflammatory cytokine secretion. Cytometric bead array (CBA) was performed to measure cytokine levels in supernatant of LMP1-transfected DC. LMP1-DC secreted significantly higher levels of IL-6, IL-8, TNF-α, and IL1-β (Fig. 3.3B). Surprisingly, LMP1 did not induce secretion of IL-12p70. LMP1, being a viral mimic of CD40 and potent inducer of IFN signaling activity, was expected to induce IL-12p70. Previously in the literature it has
been shown that the combination of CD40 and IFN signaling is sufficient to induce IL-12p70 secretion in MoDC [26, 79]. However, no IL-12p70 secretion was observed following LMP1 electroporation.

3.1.4. Human MoDC in vitro migration

For DC to be effective therapeutic agents, they must possess the ability to migrate to the draining lymph node upon injection. Previous studies have shown that PGE2 is required for MoDC migration [65, 66]. We decided to test different combinations of maturation components commonly used for DC maturation to determine if any could overcome the requirement for PGE2. Consistent with the literature, we observed that only DC cultured in the presence of PGE2 migrated towards CCL19 when compared with alternative DC maturation strategies (Fig. 3.4A).

To determine the effects of LMP1 on DC migration, human MoDC were electroporated with LMP1 mRNA and cultured for 48 hours before evaluating migration toward CCL19. It was found that LMP1 alone did not enhance DC migration compared to control GFP transfected DC (Fig. 3.4B). Surprisingly, when LMP1-DC were matured with Mimic cytokines in the absence of PGE2, migration was increased to levels significantly higher than that of either LMP1-DC or Mimic-DC.

3.1.5. B16-F10 dendritic cell tumor immunotherapy model

Next we determined whether LMP1 could enhance a dendritic cell cancer immunotherapy model. A B16-F10 tumor cell line was chosen for this assay due to its
Figure 3.3: LMP1 electroporation enhanced maturation, activation, and cytokine production of human monocyte-derived dendritic cells. Human MoDC were electroporated with LMP1, or GFP mRNA. Mimic was added as a positive control. (A) After 48 hours, DC were stained for maturation markers, and analyzed by flow cytometry. (B) Supernatant was collected after 2 days and cytokine measured by CBA.
Figure 3.4: Electroporation of LMP1 enhances human MoDC migration without the addition of PGE2. Monocytes were isolated from buffy coat and cultured in the presence of GM-CSF and IL-4 for 5 days to generate DC. (A) On day 5 DC were matured with the indicated combination of Mimic, PGE2, LPS, or CD40L. (B) On day 5, MoDC were electroporated with LMP1 or GFP mRNA. Mimic cytokine cocktail was added as a positive control. LMP1+Cytokines DC were cultured with IL-6, IL-1β, and TNF-α at the same concentration as Mimic. On day 7 cells DC migration was measured using a transwell migration assay in response to CCL19. 1.5x10⁵ DC were allowed 90 minutes to migrate.
aggressive nature and the limited efficacy of current cancer vaccine therapies in this model. It was reasoned that if any significant changes were observed in tumor growth or survival, this would be indicative of a potent dendritic cell adjuvant.

C57BL/6 mice were challenged with B16-F10 tumors in the flank and monitored for tumor onset, growth, and survival. On day 3 following challenge, animals were vaccinated with $1 \times 10^6$ DC electroporated with gp100 antigen mRNA and matured with either electroporation of LMP1 mRNA or culture with Mimic. Mice were vaccinated every 7 days for a total of 3 injections. As a gold standard treatment, $1 \times 10^6$ B16/F10 tumor cells expressing GM-CSF (GVAX) were irradiated and given s.c. every 3 days for a total of 3 injections.

It was found that mice vaccinated with LMP1-DC displayed a significant reduction in tumor growth rate compared to gp100-DC (Fig. 3.5A). Animals treated with LMP1-DC displayed tumor growth rates similar to that of our gold standard treatment, GVAX and Mimic-DC. Although LMP1-DC displayed a slower rate of tumor growth comparable to known protocols used in the clinic, there was no increased survival observed in the LMP1-DC treated animals (Fig. 3.5B).

3.1.6. IL-12p70 secretion in response to electroporation

To further investigate why LMP1 transfected dendritic cells did not secrete IL-12p70, we examined the role of electroporation on DC dysfunction. IL-12p70 plays a key role in T cell maturation and proliferation [28]. Due to the similarity of LMP1 signaling to that of the CD40 receptor along with IFN-based signaling, IL-12p70 secretion was expected [84-86].
Figure 3.5: LMP1 electroporated DC enhanced responses against B16-F10 tumors. 5x10^4 B16 tumor cells were injected intradermally into the flank of C57BL/6 mice. Three days later, mice were treated with BMDC electroporated with GP100 antigen mRNA and matured by either LMP1 mRNA transduction or Mimic cytokine cocktail. Mice were vaccinated with 1x10^6 DC injected intradermally into the flank every 7 days, starting 3 days after tumor challenge, for a total of 3 treatments. (A) Mean tumor area. (B) Survival curve.

Figure 3.6: IL-12p70 secretion from human MoDC. Immature DC were stimulated with LPS plus IFN-γ and supernatants were collected. IL-12p70 was measured by CBA. (A) Untreated DC were stimulated with LPS plus IFN-γ and/or electroporated at either 300V or 350V. Other DC were stimulated with LPS and IFN-γ after a 24 hour recovery period. (B) Immature DC were infected with Ad5-GFP at an MOI of 1,000. Post-infection, DC were stimulated with LPS+IFN-γ. Supernatants were collected at 16 and 36 hours. All cytokine analysis was done by Cytometric bead array.
Interestingly, we found that when DC were electroporated at either 300V or 350V and then stimulated with LPS and IFN-γ, DC failed to secrete IL-12p70 post electroporation (Fig. 3.6A.). However, LPS plus IFN-γ was a potent inducer of IL-12p70 in non-electroporated DC. When electroporated DC were allowed 24 hours to recover and then stimulated with LPS plus IFN-γ, IL-12p70 secretion increased but at a much lower level compared to non-electroporated DC. As previously shown in Figure 3.3B, cytokine secretion of other proinflammatory cytokines was not inhibited by electroporation. IL-12p70 appears to be uniquely inhibited by the electroporation protocol.

It has been previously published that DC have an impaired ability to secrete IL-12p70 post electroporation [96]. However, when DC are transduced using a viral vector, they maintain the ability to secrete IL-12p70. Therefore we next tested the ability of DC to secrete IL-12p70 in response to adenoviral vector transduction. Consistent with the findings of Dullaers et al., DC transduced with an adenoviral vector could be stimulated to secrete IL-12p70 (Fig. 3.6B). In fact, levels of IL-12p70 were higher when adenoviral vector transduction was combined with LPS plus IFN-γ than for LPS plus IFN-γ stimulation alone.

3.1.7. DC survival in response to electroporation

Although many groups have examined dendritic cell survival in response to electroporation [97-99], the majority of publications have only looked at 24 or 48 hours post electroporation and not at later timepoints. However, DC will typically reach the lymph node around 3-4 days post electroporation based on a 24-48 hour maturation
followed by processing and intradermal injection proximal in the patient. Therefore, to test long term survival, DC were either electroporated with mRNA or transduced with Ad5. Replicate wells were examined daily for 5 days to assess DC survival.

By 24 to 48 hours after transfection, electroporated DC had significantly decreased survival compared to both no treatment DC and Ad5 transduced DC (Fig. 3.7A.). This initial die off was most likely the result of electroporation. Importantly, electroporated DC survival continued to decline on day 3. Survival reduced from 75% to around 40%. Survival for Ad5 transduced DC was similar to the no treatment group, with survival never dropping below 80%. These findings suggest that, based upon viability, Ad5 transduction may be a superior method of transfection compared to mRNA electroporation.

3.1.8. Pmel response to transfection method

Based on the decreased survival observed in Figure 3.7A, we next evaluated which transfection method is superior at inducing an adaptive T cell response. The Pmel-1 mouse model was used to quantify the ability of gp100 antigen-loaded DC to stimulate antigen-specific T cell proliferation in vivo. Pmel mice carry a rearranged T cell receptor transgene specific for human gp100 (26). To evaluate Pmel responses, C57BL/6 mice were adoptively transferred with purified Pmel CD8+ T cells. After one day, mice were injected i.d. into the flank with DC either electroporated with gp100 mRNA or transduced with Ad5-gp100 and matured with Mimic. After 5 days, spleens
Figure 3.7: DC survival and T cell stimulation based on transfection method. (A) Immature human MoDC were either electroporated at 300V or transduced with Ad5 (performed in triplicate). Every 24 hours survival was assessed by trypan blue staining. (B) Immature mouse BMDC were either electroporated with gp100 mRNA or transduced with Ad5-gp100. Mimic was used for maturation as indicated. The next day, 1x10^6 DC were injected intradermally into the flank of C57BL/6 mice adoptively transferred 24h previously with 1x10^6 CD8+ T cells isolated from a Pmel-1 transgenic mouse. For positive control, LPS and gp100 peptide was injected subcutaneously. 5 days following DC injection, splenocytes were stained for CD3, CD8, and Thy-1.1. Data represented as percent CD3+ CD8+ T cells that were Thy1.1+. 
were analyzed for CD3+CD8+Thy1.1+ T cells. As shown in Figure 3.7B, Ad5 transduced DC stimulated a robust Pmel response, significantly greater than electroporated DC. Immature Ad5 transduced DC also stimulated a stronger Pmel response compared to electroporated DC matured with Mimic, suggesting that electroporation is inducing DC dysfunction. Lack of IL-12p70 secretion along with reduced survival post electroporation likely account for the difference observed.

3.2. Cloning and evaluating the effects of Ad5-LMP1 in mouse bone marrow-derived dendritic cells

3.2.1. Construction of an adenoviral vector expressing LMP1

To improve the efficacy of LMP1 expressing DC, it was decided to use an adenoviral system for LMP1 expression as an alternative to electroporation. From our previous data, electroporation-mediated DC damage may be masking the benefits of using LMP1 as a molecular adjuvant. We cloned LMP1 into a replication-defective Ad5 viral vector system for transduction of DC. Constructs were cloned such that LMP1 expression was directly linked to GFP protein expression using an internal ribosomal entry site (IRES). Initial attempts to construct Ad5-LMP1 proved difficult. Due to the stimulatory nature of LMP1, high titer viral stock could not be obtained. To prevent LMP1 expression in the packaging cell line, a 3rd generation tetracycline-inducible system was chosen. In this system a Tet-transactivator protein binding to a TRE-3G promoter upstream of the LMP1 gene to activate transcription. This system allows very tight control of LMP1 expression in the AD293 packaging cell line. When doxycycline (Dox) was added to the cell culture media, LMP1 expression was readily detectable by
Western blot (Fig. 3.8A). In the absence of doxycycline only a faint LMP1 band was observed. To further test the expression control of the tetracycline inducible system, real-time PCR was performed on AD293 cells transduced with Ad5-LMP1 in the presence or absence of doxycycline (Fig. 3.8B). pcDNA3.1-LMP1 was lipofected into AD293 cells as a positive control. Doxycycline induced high levels of LMP1 expression compared to the Dox-negative group. Levels were three times higher compared to the plasmid-transfected positive control. These findings suggest that Ad5-LMP1 expresses LMP1 at high levels.

3.2.2. Ad5-LMP1 is biologically active and induces NF-κB

Previously, the signaling activity of LMP1 DNA was tested with an NF-κB and IFN-β luciferase reporter assay (Fig. 3.2A-B). To confirm that Ad5-LMP1 has biological activity, a 293-SEAP cell line was used. These 293-SEAP cells express the gene for secreted embryonic alkaline phosphatase (SEAP) under control of an NF-κB promoter. In the presence of doxycycline, Ad5-LMP1 induced SEAP secretion in a dose-dependent manner (Fig. 3.8C). In the absence of doxycycline only low levels of SEAP were observed, consistent with the low background levels of protein expression (Fig. 3.8A).

3.2.3. Ad5-LMP1 transduction activated and matured mouse bone marrow-derived dendritic cells

Mouse BMDC were transduced with Ad5-LMP1 to determine the effect of LMP1 transduction on DC maturation. 36 hours following transduction, DC were analyzed for markers of maturation and activation by flow cytometry. DC transduced with Ad5-LMP1
Figure 3.8: Construction of Ad5-Tet-On-LMP1. AD293 cells were transduced with Ad5-Tet-On-LMP1-IRES-GFP in the presence or absence of doxycycline. 

(A) After 48 hours, cells were harvested and lysed in RIPA buffer. Proteins were denatured and run on a 4-15% SDS-PAGE gel. Protein was transferred to PVDF membrane and probed with mouse Anti-LMP1 antibody. 

(B) Real-time PCR was performed on reverse transcribed purified RNA from Ad5-transduced AD293 cells in the presence or absence of doxycycline. AD293 cells were transfected with pcDNA3.1-LMP1 plasmid as a positive control. 

(C) Viral stocks of Ad5-LMP1 were serially diluted and added to 293-SEAP reporter cells in triplicate, in the presence or absence of doxycycline. After 36h, secreted alkaline phosphatase was measured.
showed significantly higher levels of activation and maturation compared to Mimic or Ad5-GFP controls. There was also significant upregulation of CD80, CD86, CD40, CD83, and CCR7 expression (Fig. 3.9A), consistent with the ability of LMP1 to mimic CD40-mediated signaling [82].

3.2.4. LMP1 transduced mouse BMDC expressed high levels of proinflammatory cytokines

Next, we determined the effect of LMP1 transduction on DC proinflammatory cytokine secretion. Cytometric bead array (CBA) was performed to measure cytokine levels in supernatant of Ad5-LMP1 transfected DC. Consistent with the finding from electroporation of LMP1 mRNA, LMP1 transduced DC secreted significantly higher levels of IL-12p70, TNF, IFN-γ, MCP-1, and IL-6 compared to GFP transduced DC (Fig. 3.9B). Surprisingly, LMP1-DC also secreted significantly higher levels IL-12p70 in contrast to LMP1 electroporated DC. This increased IL-12p70 secretion by Ad5-LMP1 is likely related to electroporation suppressing LMP1 induced IL-12p70 secretion. As previously observed, Mimic-DC induced only low levels of IL-12p70 secretion.

3.2.5. LMP1 enhanced BMDC in vivo migration without the requirement for PGE2

For DC to be an effective therapeutic agent, they must possess the ability to migrate to the draining lymph node upon injection. To determine the migratory abilities of LMP1-DC, Ad5-LMP1 transduced DC were CFSE labeled and injected intradermally.
Figure 3.9: Ad5-LMP1 enhanced bone marrow-derived dendritic cell maturation and cytokine secretion. BMDC were transduced with Ad5-LMP1 or control virus. Mimic was added as a positive control. (A) On day 5, cells were stained for maturation markers, and analyzed by flow cytometry. (B) On day 5, supernatant was collected and analyzed using the BD mouse inflammation CBA kit.
into the flank of C57BL/6 mice. After 48 hours, draining inguinal lymph nodes were removed and cells analyzed by flow cytometry for the total number of migrated CD11c+CFSE+ DC. CFSE+ cells were readily detected by flow cytometry (Fig. 3.10A). Ad5-LMP1 significantly enhanced DC migration compared to an Ad5-GFP control and was equivalent to Mimic-DC (Fig. 3.10B). These data suggest that Ad5-LMP1 transduction can enhance in vivo migration without the requirement for PGE2.

3.2.6. LMP1 induced strong antigen-specific T-cell responses

The next critical question in evaluating LMP1 as a dendritic cell molecular adjuvant was whether or not LMP1 could enhance the ability of dendritic cells to stimulate an adaptive T cell response. The Pmel-1 mouse model was used to quantify the ability of Ad5-gp100 antigen and Ad5-LMP1-transduced DC to stimulate antigen-specific T cell proliferation in vivo. As mentioned above, Pmel mice carry a rearranged T cell receptor transgene specific for human gp100 (26). To evaluate Pmel responses, C57BL/6 mice were adoptively transferred with purified Pmel CD8+ T cells. The following day, DC were transduced with Ad5-gp100 and matured with either Ad5-LMP1 or Mimic and injected i.d. into the flank of C57BL/6 mice. After 5 days, spleens were analyzed for CD3+CD8+Thy1.1+ T cells. As shown in Figure 3.11, LMP1-DC stimulated a robust Pmel response, significantly higher than both immature DC and Mimic-DC. PMEL cells were approximately 7.5% of all circulating CD8+ T cells, suggesting that LMP1-matured DC were highly effective antigen presenting cells (Fig. 3.11).
Figure 3.10: Ad5-LMP1 transduction enhanced mouse bone marrow-derived dendritic cell migration. BMDC were transduced with Ad5-LMP1-IRES-GFP or Ad5-GFP. Mimic was added as a positive control. After 36 hours, DC were CFSE labeled and 5x10^5 DC were injected intradermally into the flank of C57BL/6 mice. After 48 hours inguinal lymph nodes were processed for single cell suspension. Migrated cells were defined as CFSE+ CD11c+ by flow. (A) Flow plots of CSFE positive DC. (B) Comparison of LMP1-DC to Mimic and GFP control.
Figure 3.11: Ad5-LMP1 transduction enhanced mouse bone marrow-derived dendritic cell T cell stimulation. BMDC were transduced with Ad5-GP100. 24h later, DC were transduced with Ad5-GFP, Ad5-GFP plus Mimic cytokines, or Ad5-LMP1. The next day, 1x10⁶ DC were injected intradermally into the flank of C57BL/6 mice adoptively transferred 24h previously with 1x10⁶ CD8+ T cells isolated from a Pmel-1 transgenic mouse. For positive control, LPS and gp100 peptide was injected subcutaneously. 5 days following DC injection, splenocytes were stained for CD3, CD8, and Thy1.1. Data represented as percent CD3+ CD8+ T cells that were Thy1.1+. 
3.2.7. LMP1-DC slowed B16-F10 tumor growth, delayed tumor onset, and increased survival

Based on the ability of Ad5-LMP1 transduced DC to induce an adaptive T cell response, we next wanted to determine whether this would translate into a more effective dendritic cell based immunotherapy. To answer this question we turned to the B16-F10 melanoma model. We had previously shown that LMP1 electroporated DC were effective at slowing tumor growth in a B16-F10 model (Fig. 3.5A). This tumor challenge model was repeated to assess Ad5-LMP1 transduced DC. C57BL/6 mice were challenged with B16-F10 tumors in the flank and monitored for tumor onset, growth, and survival. On day 3 after challenge, animals were vaccinated with $1 \times 10^6$ DC transduced with Ad5-gp100 antigen and matured with either Ad5-LMP1 or culture with Mimic. Mice were vaccinated every 3 days for a total of 6 injections. As a gold standard treatment, $1 \times 10^6$ B16-F10 tumor cells expressing GM-CSF (GVAX) were irradiated and given s.c. on the same vaccination schedule as DC immunotherapy.

Mice vaccinated with LMP1-DC displayed a significant reduction in tumor growth rate compared to all other treatment groups (Fig. 3.12A). LMP1 also significantly delayed the onset of tumors compared to all other groups (Fig. 3.12B). Palpable tumors did not appear until day 15 compared to day 7-10 with other treatments. LMP1-DC showed significantly improved survival compared to GVAX (Fig. 3.12C). LMP1-DC survival also approached statistical significance compared to gp100-DC and Mimic-DC (p=0.0707 and p=0.0825 respectively). These findings suggest that LMP1 has the potential to enhance DC therapeutic vaccines. These data also suggest that Ad5-LMP1 transduction of dendritic cells yields a more immunostimulatory DC compared to Mimic.
3.2.8. LMP1-DC slowed tumor growth in a lung metastasis model

To follow up on the findings of the DC therapy flank tumor model, it was decided to test LMP1 in a lung metastasis model to evaluate the ability of LMP1-DC to prevent establishment and/or growth of metastatic lesions. C57BL/6 mice were injected i.v. with B16-F10 tumor cells. On day 3 after challenge, animals were vaccinated s.c. with $1 \times 10^6$ DC transduced with Ad5-gp100 antigen and matured with either Ad5-LMP1 or Mimic. Mice were vaccinated every 3 days for a total of 6 injections. As a gold standard treatment, $1 \times 10^6$ GVAX cells were irradiated and given s.c. on the same vaccination schedule as DC immunotherapy. Lungs were harvested at day 21 post tumor injection. Overall, mice treated with Ad5-LMP1 transduced DC showed reduced tumor burden compared to GFP-DC and the no treatment group based on visual observation and lung weight (Figs. 3.12D and E). GVAX and Mimic matured DC were moderately effective at reducing tumor burden, but did not reach statistical significance by lung weight (Fig. 3.12E). Of the animals treated, only 2 animals had no visible tumor lesions; One animal from the LMP1 treatment group and one from the Mimic treatment group. These data are consistent with our flank tumor models findings (Fig. 3.12A). Overall, LMP1 appeared to be an effective adjuvant to mature DC for tumor immunotherapy.

3.2.9. Vaccination with LMP1-DC enhanced protection from vaccinia-Gag challenge

Although LMP1 appears to enhance DC immunotherapy in tumor models via T cell mediated immunity, we wished to evaluate directly the ability of LMP1 to induce an effective CD8+ T cell response. It was decided that LMP1-DC would be tested as a
prophylactic vaccine for a viral challenge model using vaccinia-Gag. For this model, mice were vaccinated only once with $1 \times 10^5$ DC transduced with Ad5-Gag antigen and matured with either Ad5-LMP1 or Mimic. After 4 weeks, mice were challenged i.p. with vaccinia-Gag virus. 5 days after challenge, ovaries were titered for virus. We observed a mean 5-log reduction in viral titers in mice vaccinated with LMP1-DC compared to no treatment (Fig. 3.13). 4/6 mice vaccinated with LMP1-DC showed undetectable viral titers, compared to 2/5 animals vaccinated with Mimic-DC. We also observed a statistically significant reduction in viral titers between GFP-DC and LMP1-DC (p<0.05). Although there was no significant difference comparing LMP1-DC to Mimic matured DC, there was a trend to lower viral titers. These findings suggest that LMP1 is effective at priming a CD8+ T cell anti-viral immune response. The effectiveness of LMP1-DC for treating a chronic viral infection like HIV is yet to be determined. However, these data support the concept that LMP1-DC may be effective for viral immunotherapy in addition to cancer immunotherapy.

3.3. Evaluating the effects of Ad5-LMP1 in human monocyte-derived dendritic cells

3.3.1. Ad5-LMP1 matured and activated human monocyte-derived dendritic cells

Based on our promising mouse data, we next evaluated Ad5-LMP1 transduction of human MoDC. Ad5-LMP1 transduced human MoDC showed classic morphological signs of activation, including increased cell clumping compared to Ad5-GFP transduced DC (Fig. 3.14A). Similar to mice, human DC transduced with LMP1 displayed a significant upregulation of activation markers including CD80, CD86, CD83, CCR7,
Figure 3.12: LMP1-DC enhanced responses against B16-F10 tumors. 5x10⁴ B16 tumor cells were injected either intradermally into the flank (A-D) or i.v. (E,F) into C57BL/6 mice. Three days later mice were treated with BMDC transduced with Ad5-GP100 antigen and matured with either Ad5-LMP1 transduction or Mimic. Mice were vaccinated with 1x10⁶ DC injected intradermally into the flank every 3 days, starting 3 days after tumor challenge, for a total of 6 treatments. (A) Mean tumor area. (B) Individual tumor growth curves. (C) Tumor appearance survival curve. (D) Mouse survival curve. (E) Images of lung tumors at day 21. (F) Lung weight.
Proinflammatory cytokine secretion was also significantly increased (Fig. 3.14C). LMP1 induced cytokine secretion that either peaked by 12-24 hours after transduction, such as TNF and IL-8, or gradually increased during a 48-hour culture, such as IL-6, IL-1β, and IL-12p70. Surprisingly, unlike mouse DC, human MoDC secreted low levels of IL-12p70 following transduction with Ad5-LMP1.

Next, we evaluated the ability of LMP1-DC to respond to restimulation. Although LMP1 is a potent activator of DC, we set out to determine if this activation leads to dendritic cell exhaustion similar to other forms of maturation (i.e. LPS plus IFN-γ or Mimic). To answer this question, DC matured with LMP1 or control DC were restimulated the next day with LPS plus IFN-γ. After 24 hour, supernatants were analyzed for cytokine secretion. Following restimulation, LMP1-DC secreted high concentrations of IL-12p70 and TNF at levels approaching those observed following stimulation of immature or Ad5-GFP transduced DC (Fig. 3.14D). High levels of IL-12p70 and TNF from immature and Ad5-GFP DC were anticipated given that LPS plus
IFN-γ served as a primary stimulation. In contrast, Mimic-DC or LPS plus IFN-γ matured DC showed low levels of IL-12p70 and TNF secretion following restimulation. This is consistent with reports of DC exhaustion following Mimic maturation [78, 79]. In contrast, LMP1 maturation did not lead to dendritic cell exhaustion despite effective activation and maturation of the DC.

### 3.3.2. Ad5-LMP1 transduction enhanced migration of human MoDC in vitro

To assess migration of human DC following Ad5-LMP1 transduction, we used a CCL19 transwell migration assay. Previously we have shown that electroporation of LMP1 mRNA into MoDC did not lead to increased dendritic cell migration. However, when DC were electroporated with LMP1 and matured with IL-1β, TNF-α, and IL-6, DC migration was 2-fold higher compared to Mimic-DC (Fig. 3.4B). We decided to repeat these experiments using Ad5-LMP1 transduction. DC were transduced with Ad5-GFP or Ad5-LMP1 and matured with or without Mimic for 48 hours before evaluating migration toward CCL19. Ad5-LMP1 significantly enhanced DC migration compared to GFP-DC (Fig. 3.15), but at modest levels compared to Mimic-DC. Consistent with our findings with LMP1 electroporation, when LMP1-DC were further matured with Mimic cytokines in the absence of PGE2, migration was increased to levels significantly higher than those of either LMP1-DC or Mimic-DC.

### 3.3.3. Ad5-LMP1 enhanced T cell stimulation in a mixed leukocyte reaction

Next, we investigated the T cell immunostimulatory activity of LMP1-transduced human MoDC using a mixed leukocyte reaction (MLR). DC were transduced and
matured prior to coculturing with CFSE-labeled allogeneic PBMC for 7 days. CD4+ and CD8+ T cell proliferation were analyzed by staining for T cell markers. We observed a significant increase in CD4+ and CD8+ T cell proliferation for LMP1-DC compared to
Figure 3.14: Ad5-LMP1 enhanced maturation, cytokine production, and migration of human monocyte-derived dendritic cells. Human MoDC were infected with Ad5-LMP1-ires-GFP or Ad5-GFP. Mimic was added as a positive control. Cells were then cultured for 48 hours (A) Images of DC morphology. (B) DC were stained for maturation markers, and analyzed by flow cytometry. LMP1 transduced DC were gated based on GFP expression. (C) Supernatant was collected every 12 hours for 2 days and cytokine measured by CBA. (D) After 48-hour maturation, DC were washed and restimulated with LPS and IFN-γ. Supernatants were collected after 24 hours. All samples were analyzed using the BD human inflammation CBA kit.
untreated or GFP-DC (Fig. 3.16A). Similarly, Mimic-DC showed high levels of CD4+ and CD8+ T cell proliferation, which was significantly higher compared to LMP1-DC.

### 3.3.4. Ad5-LMP1 transduction enhanced anti-Gag T cell responses in HIV+ patient DC:T cell cocultures

To further address the issue of T cell stimulation of LMP1-DC using an ex vivo model, we evaluated the ability of DC to stimulate Gag-specific T cell responses in PBMC from virally-suppressed (<50 copies/ml) HIV+ patients on antiretroviral therapy. DC generated from patient monocytes were cultured with autologous lymphocytes following transduction of DC with Ad5-Gag and maturation with either Ad5-LMP1, Mimic, or Ad5-GFP as a negative control. LMP1-DC induced significantly higher IFN-γ and IL-2 ELISPOT responses to a Gag peptide pool compared to untreated lymphocytes or lymphocytes cocultured with GFP-transduced DC (Fig. 3.16B). Although we observed no significant difference between LMP1-DC and Mimic-DC, there was a trend toward higher Gag responses with Mimic-DC.
Figure 3.15: LMP1 enhanced DC migration without the addition of PGE2.
Monocytes were isolated from buffy coat and cultured in the presence of GM-CSF and IL-4 for 5 days to generate DC. On day 5, Human MoDC were infected with Ad5-LMP1-IRES-GFP or Ad5-GFP. Mimic cytokine cocktail was added as a positive control. On day 7 cells DC migration was measured using a transwell migration assay in response to CCL19. 1.5x10^5 DC were allowed 90 minutes to migrate. LMP1+Cytokines were given IL-6, IL-1β, and TNF-α at the same concentration as Mimic without the addition of PGE2.
Figure 3.16: Human MoDC Allo-MLR and HIV+ DC:T cell coculture. (A) MoDC were transduced with Ad5-LMP1-IRES-GFP, Ad5-GFP, or matured with Mimic. On day 7, DC were cocultured with CFSE labeled allogeneic lymphocytes at a ratio of 10,000DC:100,000 lymphocytes. After 7 days, cells were stained for T cell markers and analyzed by flow cytometry. (B) DC were transduced with Ad5-Gag and matured with either Ad5-LMP1-IRES-GFP or Mimic. On day 7, DC were cocultured with patient lymphocytes at a ratio of 10,000DC:100,000 lymphocytes. After 12 days, cells were analyzed by IFN-γ and IL-2 ELISPOT assay in response to a Gag-peptide pool.
Chapter 4- Discussion

Immunotherapy with autologous monocyte-derived dendritic cells is a promising treatment approach for cancer and HIV. Studies have demonstrated that DC therapy can increase circulating CD4+ and CD8+ T cells, induce immunity against loaded antigen, and in some patients increase survival [41, 54, 60]. However, to date DC immunotherapy clinical trials have only shown modest clinical efficacy, possibly due to suboptimal or dysregulated maturation and activation of the DC [36, 46]. The dysregulation induced by PGE2 [27, 67, 68, 79] and the requirement for PGE2 to induce DC migration to the local draining lymph node is of particular interest to us [65, 66]. We hypothesized that the viral protein LMP1, a constitutively active CD40 mimic, would more effectively activate and mature DC compared to cytokine cocktails containing PGE2. LMP1 activates DC without the need for external ligation, providing an alternative to CD40 [88]. LMP1 is also appealing from a clinical standpoint because costly GMP grade cytokines would not be required to mature the DC, and LMP1 encoded in RNA or viral vector could be easily incorporated into existing RNA or viral vector antigen delivery strategies.

Initial luciferase and SEAP assays (Fig. 3.2A-B & Fig. 3.8C) showed that LMP1 was a potent inducer of NF-κB and IFN-β in vitro. mRNA electroporation data showed that LMP1 could effectively mature dendritic cells. Similarly, levels of key maturation and activation markers such as CD40, CD80, CCR7, and CD83 were upregulated, suggesting the DC will be able to provide superior T cell stimulation. Electroporation of DC with LMP1 also resulted in the secretion of several proinflammatory cytokines, with the notable exception of IL-12p70. Although LMP1 electroporation also did not lead to increased migration rates in DC, when LMP1-electroporated DC were cultured with
Mimic cytokines without PGE2, LMP1-DC migrated at levels 2-fold higher than DC matured with Mimic cytokine cocktail containing PGE2 (Fig. 3.4B). These findings suggest that LMP1 may be a useful replacement for PGE2 for DC therapy, in particular, when combined with the Mimic cytokines IL-6, IL-1β, and TNF. Previous literature suggests that PGE2 is necessary to induce functional migration of ex vivo DC to the lymph node [65, 66]. However, our data provide evidence that LMP1 can be used to induce similar levels of DC migration compared to PGE2, while avoiding the previously mentioned side effect of PGE2.

We next evaluated the efficacy of LMP1 matured DC for cancer immunotherapy using a B16-F10 cancer model. The reduction in growth of this aggressive tumor by LMP1-DC highlights the potential of LMP1 as a DC immunotherapy adjuvant. LMP1 significantly slowed tumor growth compared to DC electroporated with gp100 alone. Tumor growth was similar when compared to Mimic-matured DC and the GVAX gold standard control. Although tumor growth was slowed, this reduction did not lead to enhanced survival. We believe this may be due to DC dysfunction induced by electroporation as opposed to a lack of enhanced stimulation by LMP1.

Given the similarity of LMP1 signaling to that of the CD40 receptor, the lack of IL-12p70 secretion was unexpected [85, 86]. CD40 signaling is known to induce IL-12p70 secretion in MoDC, especially when combined with interferon [26, 28, 79]. LMP1 is also known to provide constitutive NF-κB, MAP kinase, IRF7, and PI3 kinase signaling [84]. Together, these signals would be expected to induce robust IL-12p70 gene expression. After further investigation we determined that the cause of decreased IL-12p70 secretion was electroporation-mediated damage to the DC and not the inability
of LMP1 to stimulate IL-12p70 expression (Fig. 3.6A-B). Consistent with these findings, Dullaers et al. showed that electroporation of DC caused impairment in IL-12p70 secretion [96]. They also observed that this impairment was not present when lentivirus was used to transduce DC. Also, no evidence of preexisting immune responses to Ad5 were observed in our Ad5-GFP controls. Together these findings suggest that viral vectors may be a better delivery system for expression of LMP1 in dendritic cells. Consistent with this, we observed that DC could be efficiently transduced with adenoviral vectors while maintaining the ability to secrete IL-12p70 (Fig. 3.6B).

Electroporation had additional detrimental effects on monocyte-derived dendritic cells. When a long term DC survival assay was performed, we observed that electroporation reduced DC viability at 48-72 hours following electroporation compared to untreated controls (Fig. 3.7A). Many groups have previously reported good viability of MoDC post electroporation [97-99]. However, these studies examined viability within 24-48 hours post electroporation. When used in the clinic, DC would be expected to reach the lymph node approximately 48-72 hours following electroporation. Based on our data, a large portion of these cells will have undergone apoptosis and be unable to stimulate an immune response. Whether this cell death would be immunogenic is yet to be determined.

To evaluate whether Ad5 transduction or mRNA electroporation produce a more immunostimulatory DC, both methods were tested in a Pmel transgenic mouse model. We observed that Ad5 transduction induced higher levels of Pmel CD8+ T cell proliferation compared to electroporation. We propose that this difference in T cell
proliferation is due to the decreased survival and lack of IL-12p70 secretion observed in electroporated DC.

Because Ad5 was superior at inducing T cell proliferation in the Pmel model, we decided to further explore Ad5 transduction of LMP1 into dendritic cells. Ad5 transduction alone (Ad5-GFP) did not induce DC maturation, activation, or cytokine production (Fig. 3.9A-B & Fig. 3.14B-C). We also did not observe a significant difference between Ad5-GFP DC and the no treatment DC. This lack of activation by Ad5 vector alone made Ad5-LMP1 an ideal system to study LMP1-mediated maturation and activation of DC.

In our initial experiments LMP1 was cloned into a standard Ad5 viral vector with LMP1 expression controlled by the CMV promoter. Because LMP1 is immunostimulatory, inducing both NF-κB and type I IFN signaling [82, 84, 100], it proved difficult to obtain high titer virus using this standard Ad5 approach. It was therefore necessary to clone LMP1 into a 3rd generation Tet-inducible viral vector. This system used a Tet-transactivator protein binding to a TRE-3G promoter upstream of the LMP1 gene to activate transcription. This system is less “leaky” compared to a traditional Tet-repressor system that uses the Tet repressor to block gene expression. This 3rd generation Ad5-Tet system allowed tight control of LMP1 expression in the packaging cell line, while permitting robust expression in the target cell when doxycycline is present (Fig. 3.8A-C).

When tested in both human and mouse, Ad5-LMP1 was highly effective at maturing and activating dendritic cells (Figs. 3.9A and 3.14B). Similar to results with LMP1 electroporation, Ad5-LMP1 induced secretion of a number of pro-inflammatory
cytokines (Figs. 3.9B and 3.14C). Of particular interest was the secretion of IL-12p70 by Ad5-LMP1 transduced DC due to the central role IL-12p70 plays in T cell proliferation and maturation [28]. It has been previously shown that DC maturation in the presence of PGE2 leads to exhaustion and an inability to secrete IL-12p70 following restimulation by T cells [27, 76, 79]. For mouse DC, Ad5-LMP1 transduction was sufficient to induce secretion of large quantities (>500 pg/ml) of IL-12p70 (Fig. 3.9B). For human DC, Ad5-LMP1 induced secretion of only low levels (5-10 pg/ml) of IL-12p70 (Fig. 3.14C). However, human DC transduced with LMP1 secreted high levels (>3,000 pg/ml) of IL-12p70 following restimulation (Fig. 3.14D). This high level of secretion was not observed in Mimic-matured DC following restimulation. These findings have important implications for DC immunotherapy. LMP1-matured DC injected into a patient would be expected to secrete large quantities of IL-12p70 upon stimulation by CD40L-expressing T cells in the lymph node. This restimulation would be expected to lead to a synchronized immune response when compared to Mimic-matured DC, which would be unable to secrete IL-12p70 following CD40L stimulation due to PGE2 induced dysfunction. This inability of DC exposed to PGE2 to secrete IL-12p70 is expected to reduce the overall immune response generated in the lymph node. While PGE2 is required to bring DC to the lymph node, these DC will be suboptimal when compared to LMP1-matured DC.

To date, PGE2 has been a necessary component of DC maturation cocktails to promote migration to the lymph node [65, 66]. Alternative DC maturation protocols that do not contain PGE2 can also induce migration [72, 73]. However, migration rates induced by these alternative strategies, while above those of immature
DC, typically fail to approach the level of migration induced by Mimic. In contrast, Ad5-LMP1 induced in vitro migration of human DC and also enhanced in vivo DC migration in a mouse model (Fig. 3.10B & Fig. 3.15). Culturing human LMP1-DC with IL-6, IL-1β, and TNF-α further enhanced migration to higher levels than Mimic-DC (Fig. 3.15). Similar findings were also observed with LMP1 mRNA electroporation (Fig. 3.4B). Overall these data support the use of LMP1 as a replacement for PGE2, either alone or in combination with IL-6, IL-1β, and TNF-α. We propose that LMP1 is one of the few methods able to induce lymph node migration at levels equal or superior to PGE2.

In our studies, LMP1 enhanced adaptive immune responses in both human and mouse models, consistent with the in vitro maturation and activation activity we observed. LMP1-DC stimulated a robust T cell response in a Pmel mouse model (Fig. 3.11). Pmel+ T cell levels reached 7.5% of all circulating CD8+ T cells. These levels were similar to a LPS plus peptide positive control. LMP-DC also slowed tumor growth for both subcutaneous and metastatic B16-F10 tumors (Fig. 3.12A-E). LMP1-DC slowed the appearance of palpable tumors to day 15 compared to day 7-10 observed in other treatments. Despite this enhanced anti-tumor response, LMP1-DC were unable to fully regress tumors. However, considering the aggressive nature of B16-F10 melanoma [101], these findings do support the hypothesis that LMP1-DC induced a strong anti-tumor immune response.

Similarly, LMP1-DC generated a 5-log reduction in vaccinia-Gag viral titers compared to untreated animals in a prophylactic vaccine challenge model (Fig. 3.13). Despite a single injection of a relatively small number of DC, vaccination was highly effective. 4 out of 6 animals that received LMP1-DC showed undetectable viral titers.
days post-challenge. Mimic-DC gave a 2.5-log reduction in viral titers. Consistent with our findings in the Pmel and tumor immunotherapy models, LMP1 transduction appeared to generate a dendritic cell capable of stimulating a superior antiviral immune response. Whether LMP1 will be an effective adjuvant for DC-based immunotherapy for chronic viral infections such as HIV is yet to be determined.

An important concern for LMP1 is the oncogenic nature of the protein. LMP1 is involved in the transformation of B cells following EBV infection [80-82, 85, 102]. Since ex vivo monocyte-derived dendritic cells are non-dividing cells, the risk of oncogenesis is minimal. Nevertheless, LMP1 was cloned into a replication incompetent Ad5 vector for transduction of DC. Since the Ad5 genome remains episomal, it is safer than viruses that integrate in the genome, such as a lentiviral vector. In addition, adenoviral vectors have been previously used for dendritic cell therapy and shown to be safe in clinical trials [103, 104]. Based on these previous results, we felt confident in using Ad5 as an initial model for DC immunotherapy.

To further decrease the risks of LMP1 oncogenesis, it was put under the control of a Tetracycline inducible promoter. Unlike older systems that use Tet-repressors to block transcription of a gene, this 3rd generation system used to clone Ad5-LMP1 utilizes a Tet-transactivator for LMP1 expression. This system offers tight control over expression with minimal leakiness. We demonstrated this by real time PCR, western blot analysis, and SEAP reporter assay (Fig. 3.8A-C). In a clinical setting using LMP1-DC, DC would be initially matured in the presence of doxycycline and injected into a patient. However, following injection into the patient and in the absence of doxycycline treatment, LMP1 expression would stop. This should further reduce the risk of oncogenicity.
In future, to address concerns with the use of LMP1 in dendritic cell vaccines, other approaches can be taken to further limit the risks of oncogenesis. One approach to remove the risk of stable integration of LMP1 is to use RNA. One option is to use a non-integrating RNA virus (alphavirus or LCMV) for LMP1 transduction. These viruses remain as RNA throughout their life cycle, avoiding any production of LMP1 DNA. Another option is nanoparticle delivery of mRNA encoding LMP1. Because RNA has a short half-life, protein expression would be transient. These approaches would avoid the risk of integration into the host genome and should minimize the risk to the patient of transformation of DC or other cells.

In summary, LMP1 is a promising molecular adjuvant candidate for DC immunotherapy, either alone or in combination with cytokines such as IL-6, TNF-α, and IL-1β. LMP1 matures and activates DC, induces DC cytokine secretion, and enhances DC migration. We observed in vivo efficacy in mice, including reduced tumor growth and increased survival. LMP1-DC also showed ex vivo efficacy in a human DC:T cell coculture assay. This study highlights the potential of LMP1 as a gene-based molecular adjuvant for DC immunotherapy and as a promising alternative to current maturation protocols for DC immunotherapy.
References


