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GENERATION OF LONG-LIVED DENDRITIC CELLS FOR A DENDRITIC CELL-BASED THERAPEUTIC HIV VACCINE

Ryan Nicholas Buensuceso

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Ryan N. Buensuceso

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CERTIFICATE OF EXAMINATION

Supervisor	Examiners
Dr. Gregory Dekaban	Dr. Ewa Cairns
Supervisory Committee	Dr. Robert Gros
Dr. Stephen Barr	Dr. Vincent Morris
Dr. Joe Mymryk	

The thesis by

Ryan Nicholas Buensuceso

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_____
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Abstract

Despite advances in therapy, acquired immune deficiency syndrome (AIDS) continues to be a global problem. New therapeutic avenues are being explored, including dendritic cell (DC)-based immunotherapy. While DCs can efficiently promote an immune response, their limited lifespan within the lymph node represents an obstacle to efficient immunotherapy.

We examined different gene transfer methods, observing lentiviral transduction to be the most effective. Transduction using generated lentiviral transfer vectors encoding M11L and EGFP were used to determine effects on cellular viability. We did not observe significant differences in viability following apoptosis induction in transduced L cells. In primary DC cultures, transduction with and without M11L did not influence DC maturation or longevity in either the short or long term, though transduction was more efficient in the immature DC population. These results demonstrate that transduction is effective for gene transfer into DCs. However, techniques for dual gene expression must be further refined.

Keywords

HIV, dendritic cell, immunotherapy, vaccines, transfection, transduction, lentivirus vector, lifespan, longevity, myxoma virus, M11L

For my family, friends, and all those who have supported me over the years

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7-AAD	7-aminoactinomycin D
AIDS	Acquired Immune Deficiency Syndrome
ANOVA	analysis of variance
APC	antigen presenting cell
BMDC	bone marrow-derived dendritic cell
BSA	bovine serum albumin
CCR7	'C-C' chemokine receptor 7
CD4	T helper cell surface marker
CD8	CTL expressed coreceptor for T cell receptor
CD11c	αX leukocyte integrin α subunit
CD28	T cell co-stimulatory receptor
CD80	B7.1; costimulatory CD28 ligand
CD86	B7.2; costimulatory CD28 ligand
CMV	cytomegalovirus
CpG	TLR ligand; 'C-phosphate-G' linear sequence of bases
CTL	cytotoxic T lymphocyte
CXCR4	'C-X-C' chemokine receptor 4

DC	dendritic cell
DC-SIGN	dendritic cell-specific intercellular adhesion molecule-3-Grabbing non-integrin
DEAE	diethylaminoethyl
DEC205	lymphocyte antigen 75
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
EGFP	enhanced green fluorescence protein
FDA	Food and Drug Administration
FSC	forward scatter
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GM-CSF	granulocyte macrophage colony-stimulating factor
HAART	highly active antiretroviral treatment
HBSS	Hanks' buffered saline solution
HIV	Human Immunodeficiency Virus
HLA	human leukocyte antigen
HTLV	Human T cell leukemia virus
IFN	interferon

IL	interleukin
IRES	internal ribosome entry site
LAV	lymphadenopathy virus
LTNP	long-term non-progressor
LTR	long terminal repeat
MHC	major histocompatibility complex
MOI	multiplicity of infection
nNRTI	non-nucleoside reverse transcriptase inhibitor
NRTI	nucleoside reverse transcriptase inhibitor
PAMP	pathogen associated molecular pattern
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	phycoerythrin
PerCP	peridinin chlorophyll protein complex
PGE2	prostaglandin E2
PGK	phosphoglycerate kinase
Poly I:C	polyinosinic:polycytidylic acid

RPMI 1640	Roswell Park Memorial Institute 1640 medium
SIV	simian immunodeficiency virus
SSC	side scatter
STS	staurosporine
TLR	toll-like receptor
TNF	tumor necrosis factor
VSV	vesicular stomatitis virus

Overview

Since the discovery of acquired immune deficiency syndrome (AIDS) in the 1980s, it has evolved from a disease associated primarily with the homosexual population to a global pandemic. According to the 2010 World Health Organization AIDS epidemic report, approximately 33.3 million people worldwide are living with Human Immunodeficiency Virus (HIV). In 2009, 1.8 million deaths were attributed to AIDS while 2.6 million new cases were described (1).

The increase in people living with HIV can be in part attributed to improvements in therapy; as of 2008, 42% of infected individuals had access to therapy. This therapy does not however clear any HIV reservoirs, and interruptions in therapy are followed by rapid increases in viremia. Furthermore, various social, economical, and medical reasons result in problems in patient compliance to therapy. As such, development of an alternative means of HIV therapy is required in order to limit our reliance on antiretroviral therapy. Unfortunately, development of HIV vaccines using traditional approaches has been ineffective to date. As a result, alternative methods are being assessed.

Introduction

Human Immunodeficiency Virus

HIV, the causative agent of AIDS, was first isolated in 1981 (2). Initially, it was characterized by two independent groups as a retroviral infection caused by a virus similar to human T cell leukemia virus (HTLV)-1, and causing

lymphadenopathy and physical weakness. The virus was termed HTLV-III by the group of Robert Gallo (3), and lymphadenopathy-associated virus (LAV) by the group of Luc Montagnier (4). Further studies eventually revealed that the two viruses were indeed the same, and in 1986, they were named HIV. The virus was classified into the genus *Lentivirus*, part of the Retroviridae family.

The virus possesses a number of both structural and non-structural genes that allow it to subvert an anti-HIV immune response and to persist in the body. These genes are all encoded on the genomic positive-sense RNA, which exists as a homodimer within a mature virion. The three main regions of the HIV genome are termed *gag*, *pol*, and *env (5)*. The *gag* region encodes proteins that are responsible for providing the physical infrastructure of the virus. This infrastructure includes capsid and nucleocapsid proteins that comprise a conical shell that encases the genomic RNA homodimer (6). The *pol* gene encodes the viral enzymes reverse transcriptase, integrase, and the protease that cleaves newly synthesized polyproteins. The *env* region encodes gp160, the precursor to the envelope proteins gp120 and gp41. Also within the HIV genome are coding regions for different non-structural proteins. Regulatory proteins such as *tat* and *rev* are responsible for a number of steps in the viral life cycle, ranging from increasing the level of transcription of HIV RNA to manipulation of viral nucleic acid export (7, 8). Lastly, other accessory proteins such as *vpr*, *vif*, *nef*, and *vpu* can function to subvert normal host immune function and cause the release of newly synthesized viral particles (9).

Infection by HIV begins by transmission of the virus through several identified routes. The most common of which is through sexual contact where secretions from an infected individual come into contact with mucosal membranes of uninfected persons (10). However, transmission through blood and blood products is also common, such as through practices of needle sharing among intravenous drug users or bad hygienic practice including the reuse of needles (11). Infected mothers can also transfer the virus vertically to the fetus or during pregnancy (12, 13). Following transmission, the virus attaches to the target cell via an interaction between gp120 and host cell CD4, using CCR5 and CXCR4 chemokine receptors as a coreceptor (14). Further studies have shown, however, that gp120 is also capable of using other cell surface molecules as a receptor, including heparan sulfate, galactosyl ceramide, DEC205, and DC-SIGN. The latter two molecules are expressed on dendritic cells (DCs) (15-18).

Following binding of the virus, it then enters the cells. The predominant belief is that this occurs by fusion of the cellular lipid membrane and the lipid envelope of the virus, releasing the nucleocapsid into the target cell (19). However, more recent studies have described an alternate mechanism where bound virus is endocytosed by the cell in a clathrin dependent manner (20). Following entry, the capsid is disassembled and reverse transcriptase mediates reverse transcription of the genomic RNA into single stranded cDNA, then into double stranded linear form, and lastly into a double stranded linear form, which is imported into the nucleus and is integrated into the target cell by the viral integrase. After integration, the proviral DNA is transcribed to genomic and

subgenomic mRNA, spliced, and translated by the standard cellular machinery, leading to production of Tat and Rev. The production of Tat leads to the upregulation of proviral transcription, while Rev allows for the export of unspliced mRNA. The unspliced mRNA is used to generate Gag, which encases the full-length RNA genome, and Env polyprotein. The HIV protease processes the Env polyprotein to gp41 and gp120 subunits which are transported to the plasma membrane. Gag and Gag-Pol polyproteins associate with the HIV genomic RNA at the plasma membrane in areas enriched for the HIV Env protein as the virus begins to bud off from infected cells, ultimately released by Vpu. Maturation of the virion is completed as the viral protease processes the polyproteins into their functional forms.

Following infection, the individual enters the acute stage of infection. This is characterized by a phase of robust virus replication, accompanied with a marked drop in $CD4^+$ T cells (21). In the acute phase, $CD8^+$ T cells are weakly mobilized, which provide small amounts of control of infection (22). After several weeks, infected individuals enter the latent phase of infection. This is characterized by a steady increase in viral load and a decrease in $CD4^+$ T cell count. When $CD4^+$ T cell levels drop to below 200 cells/μL from the normal range of 800-1050 cells/μL he infected individual is said to progress to AIDS (23, 24). Here, $CD4^+$ mediated immunity is no longer effective, resulting in a large increase in susceptibility to normally innocuous infections.

Methods to counteract infection or disease progression fall into two categories, prophylactic or therapeutic. Whereas the ultimate goal in prophylaxis

is the prevention of infection, therapeutics serve to control an existing infection. To date, in the context of HIV infection, the options available in either case are limited. Currently, a number of problems inherent to HIV have prevented the formulation of an effective prophylactic treatment. The greatest hurdle to a prophylactic vaccine has been the incredible diversity of HIV (25). A number of factors contribute to the high degree of heterogeneity of HIV. Firstly, the reverse transcriptase enzyme that generates the cDNA for integration lacks proofreading ability, resulting in an error rate of 30 mutations per $1x10^6$ base pairs (26, 27). This is further enhanced by a high rate of viral turnover and by a high virus burden within an individual (28, 29). Secondly, HIV is prone to antigenic shift; coinfection with viruses belonging to different phylogenetic lines can recombine during the strand transfer involved in reverse transcription, leading to alterations in fitness and virulence (30, 31). As a result of the high variability, envelope glycoproteins that would be most accessible to neutralizing antibodies display an astonishing 20% variability in amino acid sequence within a given clade (32). When comparing isolates across clades, this number increases to greater than 35% (32). Consequently, prophylactic treatments would have to be capable of inducing an immune response against all of the different HIV subtypes across the many phylogenetic branches. Because of the difficulties in doing so, more focus has been placed on the treatment of existing infection.

Control of HIV Replication

Generally, the immune response to virus infection utilizes both a humoral and cell mediated response. The humoral response generates antibodies which

can bind to free virus particles and prevent further infection (33). The cellular response utilizes cytotoxic cells, $CD8^+$ T cells or natural killer cells, to destroy infected cells expressing viral antigens (34, 35). While the goal of a prophylactic vaccine would serve to increase the major histocompatibility complex (MHC)-II/$CD4^+$ humoral axis of the response, therapeutic vaccines should increase the MHC-1/$CD8^+$ cellular axis.

Activation of a cytotoxic T lymphocyte (CTL) response is dependent on an interaction between the $CD8^+$ T cells and a peptide-MHC-I. Upon infection, viral peptides synthesized by infected cells can be loaded onto MHC-I during transit to the plasma membrane. The peptide-MHC complex can then be presented to $CD8^+$ T cells leading to their activation and effector function. DCs, the most potent antigen presenting cell (APC) are capable of eliciting strong cellular responses. However, their interactions with $CD4^+$ T cells represent a double-edged sword, in that they can also facilitate transmission of the virus (36).

The $CD8^+$ T cell response appears to be very important in the control of lentiviral replication and disease progression. During acute infection, the CTL response follows the increase in plasma viral load (37, 38). The peak of the CTL response is concordant with a drop in virus load. A study using a primate simian immunodeficiency virus (SIV) infection model showed that depletion of CTLs during the acute phase of infection leads to failure of early HIV control (39). Transient depletion of $CD8^+$ T cells during the chronic phase of infection resulted in increased viremia, which was eventually suppressed with reappearance of $CD8^+$ T cells (40). Further evidence of the importance of $CD8^+$ T cells is

observed in long term nonprogressors (LTNPs). These individuals are infected with HIV, but maintain a very low viral titer and prolonged time before progression to AIDS. CTL levels in this group are similar to those in other HIV infected individuals; however, the difference lies in the effector capacity of the CTLs. In LTNPs, levels of perforin, a pore forming protein directed by effector CTLs towards target cells, are increased (41).

As a consequence of $CD4^+$ T cell depletion, insult to the humoral immune response is observed as well. However, in contrast to a cellular response, a neutralizing antibody response was not observed to play a role in HIV suppression in elite controllers and those on long-term highly active antiretroviral therapy (HAART)-treated patients (42). Furthermore, the generated antibody response has been observed to be unable to match the rate of virus escape and to be lacking in breadth (43, 44). This may be a result of effects of HIV on the B cell response, as the diminished responses are associated with abnormal B cell phenotypes (45). Recently, new neutralizing antibody clones have been observed following screening of 1800 infected donors, as well as a class of individuals termed 'elite neutralizers' (46, 47). These findings demonstrate that an effective neutralizing response is possible and that it may be harnessed for future therapy.

HIV Therapy

Major advances to date in the field of HIV therapy have been the development of pharmacological treatments. The first antiretroviral drugs

targeted the reverse transcriptase enzyme. These were nucleoside reverse transcriptase inhibitors (NRTIs) that competed with standard nucleotides during elongation of DNA strands (48). These nucleoside analogs lacked a 3'-OH group, preventing elongation of the cDNA strand generated during reverse transcription. Later generations of reverse transcriptase inhibitors were composed of nucleotide analogs and non-nucleoside inhibitors (nNRTIs). While nucleotide analog inhibitors function in the same manner as nucleoside inhibitors, the non-nucleoside inhibitors function by binding directly to the enzyme to inhibit catalytic activity (49). Protease inhibitors were the next class of antiretrovirals made. These drugs target the viral protease to prevent the processing of nascent polyproteins(50). This targeting prevents the functional proteins from being incorporated into assembled virions. Entry inhibitors, specifically fusion inhibitors, are one of the newest classes of antiretrovirals, with the first one being passed by the US Food and Drug Administration (FDA) in 2003 (51). These drugs function by targeting components of the infection synapse between virus and target cell. The newest class of antiretroviral drug is the integrase inhibitor, first licensed by the FDA in 2007 (52). These drugs prevent the integration of the reverse transcribed cDNA into the host genome.

These drugs, in particular NRTIs, nNRTIs, and protease inhibitors are used in HAART, the current standard of HIV therapy. Treatment with single drugs is not used, as the high mutation rate and heterogeneity of HIV within an infected individual ultimately gives rise to a drug resistant mutant. However, by using combination therapy, the probability of obtaining a progeny virion with mutations

conveying resistance to all drugs in the cocktail is considerably lower. Administration of an effective HAART regimen results in an increase in $CD4^+$ T cells and restoration of pathogen-specific immune responses. Ultimately, full recovery cannot be achieved, due in part to incomplete control of HIV replication as well as the ability of the virus to remain in various cellular reservoirs in the body (36).

It has been demonstrated that although HAART dramatically decreases the HIV plasma viral load, interruption of therapy results in a rapid return of high-level viremia (53). Reservoirs of latent virus are believed to be generated in $CD4^+$ T cells, monocytes/macrophages, DCs, and some subsets of $CD34^+$ hematopoietic stem cells early in the disease course (54-57). It is believed that the main compartment for latency is the population of resting memory $CD4^+$ T cells. Because these cells perform only the basic processes required to survive, it is likely that the virus will simply persist in the proviral form, without high levels of virus production.

Strict adherence to HAART is required to prevent viral rebound and emergence of drug resistant mutants due to reservoirs of latent virus. The International AIDS Society has recommended at least 95% adherence for optimal results in antiretroviral therapy, as modest or occasional nonadherence significantly decreases the benefits of HAART (58). However, compliance with an optimal regimen is difficult. Studies have shown common reasons for nonadherence were sleeping through dose times, difficulties in following special instructions, and changes in one's daily habits. Social issues surrounding

nonadherence include the treatment being a reminder of one's HIV positivity, others knowing of one's HIV status, and a lack of complete understanding of their own treatment (59-61). These issues are in addition to complications caused by the side effects associated with HAART and the financial burden of treatment. Although effective, the difficulties of a HAART regimen underline the need for a simpler therapeutic approach.

Vaccine Strategies

One of the major goals in the field of HIV therapy is the development of an effective vaccine. Previous attempts at doing so have made use of traditional approaches of vaccine design: live attenuated virus, whole killed virus, and protein subunit vaccines. Live attenuated virus vaccines make use of virus particles that have diminished pathogenicity and/or a reduced ability to replicate. Whole killed viruses, while also composed of intact viral particles, are non-infectious. Thus, killed viruses would be unable to infect host cells. Protein subunit vaccines are composed entirely of one or more subunits of an intact virus particle. The subunits used are often those that are normally easily accessible to neutralizing antibodies. There are also a number of novel vaccine strategies that are being assessed. These include plasmid DNA vaccines, viral vector based vaccines, and dendritic cell based immunotherapy. Regardless of the vaccine strategy used, effective $CD8^+$ T cell activation is required.

Preliminary research employing live attenuated virus vaccines in an SIV/macaque model showed that these vaccines do confer a small level of

protection (62). An advantage of this type of vaccine is that the attenuated virus is capable of infecting host cells and generating viral proteins that can be presented within the context of MHC-I, leading to the activation of a $CD8^+$ T cell response. However, long term observation demonstrated that vaccinated macaques still exhibited immune dysfunction, T cell depletion, and in some cases, progression to AIDS. Ultimately, the immunocompromised state of the host combined with the high level of replication and selective pressure can result in reversion to virulent progeny (63, 64). Consequently, this vaccine modality is not suitable for prophylactic or therapeutic purposes.

Inactivated virus vaccines have proven to be useful in preventing infection in the case of polio and influenza, but have not been effective thus far with HIV (65-67). These vaccines are produced by heat or chemical inactivation of infectious virus. One of the major difficulties in the use of this vaccine method in the context of HIV is the conservation of immunogenicity, particularly when discussing the structure of the envelope proteins (68). Nevertheless, attempts to study the feasibility of killed virus vaccines have been assessed in SIV models. Separate studies have shown that depending on the method of inactivation, these vaccines can result in a modest increase in neutralizing antibody titer, and decrease viremia if administered post-challenge (69, 70). In humans, this method was unable to generate a broad antibody response (66). Furthermore, there was an inability to promote a $CD8^+$ T cell mediated response due to a lack of antigenic epitope presentation through the MHC-I pathway.

Subunit vaccines are also often used for viruses such as hepatitis B (71). These vaccines consist of formulations of highly purified viral proteins, commonly the structural glycoproteins present within the HIV envelope. Initial trials of subunit vaccines composed of recombinant HIV gp120 have shown an increase in neutralizing antibody titer towards a homologous vaccine strain, but not against heterologous virus (72, 73). Further studies using a trimeric HIV gp140 (gp120 elongated with the gp41 ectodomain) vaccine in rabbits and macaques have shown only low levels of neutralizing antibody towards heterologous virus (74). The difficulty in all of these methods is an overall inability to account for the high level of variability in the HIV envelope glycoproteins (75, 76). To address this problem the use of cocktails of HIV envelope glycoproteins from prevalent HIV strains has been explored (77). While this method did elicit neutralizing antibody responses, further studies have to be undertaken to better understand the breadth of these responses.

DNA vaccines are composed of plasmid vectors engineered to encode immunogenic proteins. The immunogenic properties of DNA vaccines were first observed when mice vaccinated with plasmids encoding the human growth hormone gene, rather than showing increased human growth hormone production, were observed to produce antibodes targeting the hormone itself (78). However, further studies later showed that in order to obtain detectable immune responses in primates, large amounts of plasmid DNA were required. As such, research in this field is also focusing on the development of adjuvants and novel delivery methods to increase efficiency and potency.

Viral vectors have also been examined for HIV therapy for their ability to act as a vehicle of gene delivery (79-81). Vaccines utilizing viral vectors consist of viruses modified to be safe, exhibit zero to low toxicity, and encode the requisite genes, as a vehicle to deliver the genetic information. As these viruses are able to infect target cells and express the foreign proteins, the belief was that they would be able to elicit a T cell response. One of the more publicized attempts was the Merck STEP trial (82). This trial attempted to utilize recombinant adenovirus vectors to deliver the *gag*, *pol*, and *nef* genes and induce anti-HIV T cell immunity. Infection of human immune cells with the viral vectors was intended to result in expression of the viral antigens and a subsequent immune response. However, during phase III clinical trials, no protection or significant reductions in the plasma viral load post-infection was observed; rather, the vaccinated group was seen to have an increased number of new infections relative to the control group (83).

Dendritic Cell Immunobiology

DCs are the immune system's most potent and versatile APC. They are able to quickly sense inflammatory stimuli, capture antigen, transport it to secondary lymphoid organs, and mobilize antigen specific T cells (84-86). Because of their important role in immune surveillance, they localize to peripheral tissues including the skin, respiratory tract, and genito-urinary mucosa where they exist predominantly as immature cells. Here, they express low levels of surface molecules involved in the co-stimulation and activation of naïve T cells such as CD80 and CD86. However, they have a high capacity for sampling their

extracellular environment for antigens. Upon exposure to pro-inflammatory molecules such as those involved in tissue damage or pathogen associated molecular patterns (PAMPs), the DCs are activated and undergo a change in function and phenotype (87, 88). In this state, endocytic activity decreases, while co-stimulatory molecules increase. Furthermore, the migratory capacity of the DCs increases, permitting them to traffic to secondary lymphoid organs. This is mediated by an increase in cell surface chemokine receptors, notably CCR7 (89). Within the secondary lymphoid organs, the activated DCs are capable of driving Th1 differentiation through production of interleukin (IL)-12 (90-93). This permits the activated DCs to initiate an immune response along both cellular and humoral axes. Due to the level of influence that DCs have on the immune response, they have attracted attention as a therapeutic target for the treatment of disease.

In addition to the aforementioned interactions of DCs with $CD8^+$ T cells, DCs are also capable with interacting with $CD4^+$ helper T cells through an interaction with MHC-II. This leads to either cellular immunity mediated by Th1 cells, or a B cell response via the Th2 subset. The cytokine environment influences the nature of the T helper response, as Th1 responses are promoted by interferon (IFN)-γ and IL-12, whereas an IL-4 promotes a Th2 response (93, 94). Activation of Th1 cells by DCs leads to increased macrophage activity and proliferation of $CD8^+$ T cells (95). Conversely, activated Th2 generate a humoral response by interacting with antigen bound MHC-II on the surface of B cells

present in secondary lymphoid organs. The Th2 cells then deliver activating signals to the B cells resulting in their activation and proliferation (96).

Ultimately, T cell activation by DCs is dependent on their activation state. Previous research has demonstrated that DCs lacking the means to respond directly to PAMPs retain the ability to be partially activated (termed "matured") via other hematopoietic cells (97). In this scenario, other hematopoietic cells encounter pathogens and following interactions between PAMPs and the appropriate receptors, release pro-inflammatory cytokines that promote DC maturation. The matured DCs differ from activated DCs in the capacity to generate effector T cells. Both mature and activated DCs are capable of providing a costimulatory signal to resting T cells due to upregulation of CD80 and CD86, which interact with CD28 on T cells. Notably, matured DCs fail to produce IL-12, and are hence unable to drive T cell differentiation (90, 91). Furthermore, the mature DCs have been shown to be unable to prime effective $CD8^+$ T cells, resulting in a defective CTL response (98-101).

Dendritic Cell-based Therapeutics

The use of DCs in gene therapy often involves the introduction of a gene or protein product encoding a given immunogen (102). Initial attempts at antigen loading have used purified peptides, viral vectors for transduction, or antigen expressing cell extracts. Following loading, the antigen can be presented on surface MHC molecules. Proteins synthesized within the cell are loaded onto MHC-I, whereas exogeneous proteins are loaded onto MHC-II. Furthermore,

DCs are also capable of cross-presentation, where they are able to capture extracellular antigens, process them, and load them onto MHC-I for presentation to $CD8^+$ T cells. Antigen presenting DCs can traffic to regional lymph nodes, and present the loaded antigen to T cells to initiate a response (103-105). Depending on the context of antigen presentation, DCs can activate either a Th2 response leading to a humoral $CD4^+$ T cell response, or a Th1 response leading to a $CD8^+$ T cellular response.

As it is the manner in which antigen is presented by DCs that decides on either a cellular or humoral immune response, different strategies of DC preparation can be employed to generate either response. As cellular immune responses generally depend on the production of endogeneous antigen and its subsequent loading onto MHC-I, and humoral responses use exogeneous derived antigen loaded onto MHC-II, different antigen loading strategies can be used to achieve the desired response. In the former, genes encoding the antigen of interest can be transferred into DCs such that they will be able to produce the resulting protein on their own (106). Following generation of the protein, it can be processed and loaded within the cell onto surface bound MHC-I, and stimulate CTLs. Loading of MHC-II can be achieved by pulsing DCs with antigenic peptides. It has been shown that DCs pulsed *ex vivo* with cell wall constitutents from *Streptococcus pneumoniae* and administered into syngenic mice resulted in strong B cell response to capsular polysaccharides and microbial proteins of the bacterium (107). Additionally, different subsets of DCs can be used to prepare

the vaccine. It has been observed that Langerhans cells preferentially prime cellular immunity, whereas dermal DCs prime humoral immunity (108).

Engineering of DCs for use in vaccine preparations must fulfill a number of requirements. A sufficient number of DCs must be prepared for purposes of both manipulation and ensuring that enough DCs will be obtained to traffic to regional lymph nodes. They must express immunogenic peptides within the appropriate MHC context in order to initiate the correct type of immune response. Lastly, they must be activated in order to prime effector cells (109).

One of the major advances in the DC immunotherapy field was the discovery that DCs could be easily generated from precursor $CD14^+$ monocytes by culturing in the presence of IL-4 and granulocyte macrophage colony-stimulating factor (GM-CSF) (110, 111). Further refinement of this technique has allowed for the large-scale generation of commercial grade monocyte-derived DCs. It is also possible to generate myeloid DCs using $CD34^+$ hematopoietic stem cells and expand them in a similar manner, using IL-4, GM-CSF, and TNF in the culture medium (112, 113). However, the advantages of either $CD14^+$ derived DCs or $CD34^+$ derived DCs over the other in the context of HIV has yet to be studied. In addition to using either $CD14^+$ or $CD34^+$ precursors, different subsets of DCs can be generated by modifying the cytokine milieu in culture (114). These different subsets carry with them different immunostimulatory properties; however, the exact subset that would be best for HIV therapy is not clear.

The DC precursors must also be HIV-free if used to generate large populations. As $CD14^+$ and $CD34^+$ are susceptible to HIV infection, use of infected precursor cells can generate HIV infected DCs that would propagate the virus and exacerbate disease an HIV-positive individual. Furthermore, research has shown that interaction between infected DCs and infected $CD4^+$ T cells results in the stimulation of HIV replication and virion synthesis (115, 116). Because of the hazards of using infected DCs, it is important to be able to generate healthy, uninfected cells. In order to do so, uninfected precursors must be utilized. These can be cells taken from an infected individual and rigorously screened, or from an MHC-compatible, uninfected donor.

The DCs must be activated in order to achieve optimal T cell activation. Otherwise, immature DCs are unable to initiate the appropriate immune response and may be tolerogenic (117). Thus, in order to obtain potent, stimulatory DCs, they are often treated with combinations of toll-like receptor (TLR) ligands and inflammatory cytokines. A number of studies have made use of poly I:C (TLR 3 agonist), CpG (TLR 9 agonist), tumor necrosis factor (TNF)α, interferon (IFN)γ, IL-6, IL-1β, and prostaglandin E2 (PGE2) (118). The combination of cytokines and TLR ligands can mimic interactions between DCs and pathogens, to promote increases in costimulatory molecules and chemokine receptors.

Different combinations of TLR ligands and cytokines have been assessed for clinical use. It has been recently reported that the use of purified TLR ligands is not without drawbacks. Purified poly I:C, a TLR3 ligand, when used in a maturation cocktail has been shown to induce an antiviral state within the DC

(119, 120). Activation of these innate immune mechanisms may cause RNA degradation or inhibition of protein synthesis, limiting expression of introduced transgenes. To circumvent this, the use of clinical grade sources of TLR ligands has been studied, namely cocktails present in existing vaccine formulations such as the typhoid fever vaccine, a seasonal influenza vaccine, and the bacillus Calmette-Guerin vaccine. (118). The findings here show that the "vaccine matured" DCs are capable of inducing Th1 polarization, but with limited CCR7 expression. The limitation in migratory capacity was restored by supplementing with PGE2, enhancing CCR7 expression to increase responsiveness to lymph node-associated chemokines (121).

A number of studies have examined the potential to use DCs as HIV vaccines. Initial trials made use of allogeneic DCs from HIV negative individuals and autologous DCs. The DCs were loaded with either envelope gp160 or synthetic peptides corresponding to HIV-gag or pol. These DCs were injected into 6 human leukocyte antigen (HLA) identical HIV infected individuals (122). One of the recipients of allogeneic DCs demonstrated increased envelope-specific CTL and lymphocyte-proliferative responses accompanied with IFN-γ and IL-2 production. Another recipient of allogeneic DCs showed increased envelope-specific lymphocyte-proliferative response. A recipient of autologous DCs eventually showed an increase in peptide-specific lymphocyte-proliferative responses. However, in all cases, no effect on viral load was observed. Another trial used monocyte-derived DCs cultured in the presence of GM-CSF and IL-4 (123). Eighteen HIV infected individuals off HAART treatment for at least 6

months were given DCs loaded with inactivated HIV and cytokine matured. At 112 days post administration, a decrease in plasma HIV RNA was measured with 8 of the 18 recipients demonstrating a reduction of viral load of over 90%. This correlated with increased HIV-specific $CD4^+$ T cells. These results demonstrated the efficacy of a DC-based HIV vaccine to control HIV infection.

Manipulation of Dendritic Cell Lifespan

One of the key issues surrounding the use of DCs in immunotherapy is their lifespan and persistence within the lymph node (124). Furthermore, uptake of dead DCs by immature DCs may result in tolerance (125). Because the lymph node is the site of antigen presentation, it has been postulated that a limited lifespan of DCs places limits on the duration of a T-cell mediated immune response. Characterization of BrdU-labeled DCs in the absence of antigen or microbial stimuli demonstrated that they can persist anywhere between 2 and 9 days within lymphoid organs. Following antigenic and microbial stimuli however, the turnover rate increased (126). Other studies using fluorescently-labeled allogeneic murine DCs revealed that injected DCs are short lived upon migration to the lymph node persisting for upwards of 7 days, with peak levels achieved at day 2 (127). Multiple attempts have been made at circumventing shortcomings in DC lifespan. These include transfection with a number of cellular anti-apoptotic factors such as Bcl-2 and Bcl-x_L, or siRNA-mediated knockdown of the pro-apoptotic factors Bak/Bax. In the latter case, knockdown was shown to increase immune responses (128-131).

Manipulation of cellular apoptotic regulators to boost DC longevity is in itself a double-edged sword. Although they have been shown to successfully increase longevity, they may also be associated with oncogenicity, making applications involving these difficult to translate to clinical settings (132, 133). Furthermore, depending on the method of transgene introduction, effects of manipulated apoptotic factors may synergize with other proto-oncogenes whose expression may be altered following gene transfer. An alternative method makes use of apoptotic regulators that are viral in nature. The benefits of this method exist on two levels. First, it allows for the selection of regulators that are not known to have oncogenic potential. Second, as the regulators are viral and therefore foreign to the body, it is possible for the immune system to eventually eliminate cells expressing the foreign protein. This permits a temporary increase in DC longevity without resulting in overwhelming DC activity or an excessively prolonged lifespan that could contribute to cancer.

Myxoma virus encodes M11L, an anti-apoptotic protein encoded by a virus not known to be pathogenic to humans. It has shown to be important to myxoma virus pathogenicity as an M11L deletion mutant virus was markedly less pathogenic than wild type virus, being unable to cause lethal myxomatosis (134). Furthermore, the knockout virus resulted in unusual lesions with vigorous inflammatory activity, suggesting that M11L performed a function that normally compromises an effective cellular response. Research later showed that M11L was localized to the mitochondria (135). Mitochondria are central in the apoptotic pathway, where it functions in both the intrinsic and extrinsic pathways (136).

During apoptosis, the mitochondria swell, as well as changes in membrane potential. Pores formed in the mitochondrial membrane result in the release of cytochrome c and subsequent activation of caspase 9. The pores are formed by apoptotic factors Bak and Bax, while local anti-apoptotic factors, the Bcl-2 family of proteins, prevents their activation (137, 138). M11L was revealed to be a structural homolog of Bcl-2, functioning by preventing the conformational activation of Bak and Bax (139, 140) and the subsequent release of cytochrome c (141). Pharmacological induction of apoptosis in immortalized Rat2 fibroblasts infected with an M11L encoding retrovirus resulted in a 2-fold decrease in apoptotic cells over a 5 hour treatment, demonstrating its anti-apoptotic effects (135).

In the context of DC-based HIV therapy, anti-apoptotic regulators such as M11L may play a pivotal role. It has been demonstrated that the HIV envelope glycoprotein gp160 is capable of inducing apoptosis in $CD4^+$ T cells (142). Presumably, DCs engineered to express the same envelope glycoprotein would meet the same fate. However, the ability of M11L to prevent apoptosis may circumvent this problem. Previously, it was shown that inclusion of M11L in a DNA vector-based HIV vaccine was able to prevent gp140 mediated cytotoxicity, as well as increase the magnitude of a $CD8^+$ T cell response (143). This study made use of a plasmid DNA vaccine encoding both gp140 and M11L. As the ultimate fate of the vector following administration was unknown, it was postulated that the vector was endocytosed by DCs. These cells would then

synthesize and express the HIV glycoprotein while utilizing the anti-apoptotic effects of M11L to circumvent gp160 mediated cytotoxicity.

Dendritic Cell Gene Transfer

DC immunotherapy requires that the DCs are engineered to present immunogenic peptides on surface MHC. A number of different methods exist for gene transfer, generally classified as either non-viral or viral vector mediated. Commonly used non-viral mediated transfer methods include liposome or cationic-polymer mediated, and more recently, Nucleofection. Viral vectors used for transduction of cells primarily make use of either adenovirus or lentivirus.

Liposome-mediated transfection entails the use of DNA enclosed within positively charged vesicles made of a phospholipid bilayer. Upon interaction with the cell membrane, the two lipid bilayers fuse, releasing the DNA contents into the cytoplasm (144). The DNA is then imported into the nucleus, where it persists as an episome. This method is one of the most common, as it displays a high transfection efficiency in a wide variety of cell lines, and is of low toxicity to the cell. However, it does not display high transfection efficiencies in primary cells. Several groups have attempted to use this method to transfect monocyte-derived DCs, observing transfection efficiencies only as high as 10% (145).

Cationic polymers make use of charge differences between positively charged molecular polymers and negatively charged nucleic acid (146). The polymer and nucleic acid complex are endocytosed by the cell. Proton sponge effects of the cationic polymer buffer the endosomal pH and result in massive

proton accumulation and chloride influx (147). The change in osmotic pressure across the endosomal membrane eventually causes rupture of the membrane, releasing the nucleic acid. Plasmid DNA is then imported into the nucleus where it, as in lipofection, persists as an episome. Studies using this method in AG101 cells, an immortalized murine DC line, demonstrated a transfection efficiency below 1% (148).

Nucleofection is a relatively new transfection method. Rather than electroporation, where application of an electric current pulses nucleic acids into the cytoplasm, Nucleofection pulses nucleic acids directly into the nucleus (149). In the case of DNA, it can then be transcribed faster, resulting in quicker gene expression. Furthermore, Nucleofection demonstrates higher transfection efficiency across many cell types (150). A major disadvantage of the system is its proprietary nature and inability to customize parameters of the electric current.

Viral vector-mediated transduction makes use of viruses engineered to encode a gene of interest that infect the cells. In general, plasmids encoding the transgene and viral components are transfected into a mammalian cell line that packages and releases vial particles. The viral particles can then be harvested and used to transduce the cells of interest. However, as the transduction vehicles are still viral in nature, a number of safety measures are put in place to prevent pathogenicity. Often, the viruses used as vectors are either known to not cause severe pathology, or are prepared such that they are unable to do so (151).

Lentivirus transduction is a popular method of gene transfer. Importantly, because lentiviruses are capable of integration, they are well suited for infecting dividing cells and propagating engineered transgenes (152). This system utilizes three separate plasmids, a transfer plasmid encoding the integration cassette, a packaging plasmid encoding the gag, pol, tat, and rev proteins to provide the capsid structure, transcription and integration machinery (153), and *trans*-activating proteins to drive expression of the integration cassette, and an envelope plasmid that encodes the vesicular stomatitis (VSV)-G protein to pseudotype the recombinant virus. The pseudotyping of the virus permits an expanded tropism, allowing the lentiviral vector to be used for transducing a wide range of cell types (152). As the natural life cycle of lentiviruses involves the integration of the viral genome into the host genome, there are safety concerns surrounding this method. In a trial using lentiviral vectors to treat X-linked adrenoleukodystrophy, transduced $CD34^+$ hematopoietic stem cells yielded leukocytes of high polyclonality and no clustering of insertions in oncogenes or growth-related genes (154). Conversely, in a trial using lentiviral vectors to treat β-thalassemia, transduced $CD34^+$ stem cells demonstrated clonal dominance by transductants containing an activating insertion in the HMGA2 proto-oncogene (155). Although the abundance of the dominant transduced clone was stable for months after its discovery, this highlights the potential for insertional activation. It is therefore important to recognize the safety concerns of lentiviral transduction. The main concerns have been the generation of replication-competent virus and insertional activation of oncogenes, in part by viral enhancer and promoter

sequences. However, further advancement of this technology has resulted in the development of new safety measures to address some of the concerns (152, 156, 157).

Although HIV is in fact a member of the lentivirus family, a number of safety measures have been put in place in the generation of lentiviral transduction vectors. In each progressive generation of lentiviral vector, researchers have been able to dispense with a number of HIV-1 genes. Currently, only the genes absolutely required for the initial generation of the viral particle and subsequent infection are present (158). Most importantly, the newer generation lentivirus vectors are self-inactivating (157). The transfer plasmid coding for the gene of interest possesses a deletion in the 3' long terminal repeat (LTR). After assembly of the virus particles and subsequent infection into target cells, the deletion is copied into the 5' region during the reverse transcription phase of infection prior to integration. As a result, the integrated provirus lacks a complete 5' LTR capable of driving transcription and therefore is unable to support production of lentiviral transcripts capable of being packaged into progeny virions. Therefore no new infectious virus can be produced.

Dual Gene Expression

For gene therapy, it may be necessary to express multiple transgenes if more than one gene is required to establish the intended effect. Introduction of multiple genes can be achieved either by the use of multiple vectors, or by a single multicistronic vector. However, if multiple monocistronic vectors are used,

only a fraction of the target cells will acquire all of them, resulting in a heterogeneous population of unequal gene expression. For this reason, a single multicistronic vector is appealing as target cells taking up the vector will result in a largely homogeneous population of transgene-expressing cells.

Some viruses have evolved ways to initiate translation in the absence of a 5' cap, permitting translation from within an mRNA transcript. This is achieved by the presence of an internal ribosome entry site (IRES) (159). Further research has demonstrated the existence of this system within eukaryotic cells; however, the mechanism of viral IRES function is better understood (160). In general, the IRES functions by creating a scaffold within an mRNA transcript to permit protein translation independent of a 5'cap or a number of cellular translation initiation factors; the exact mechanism, however, is dependent on the origin of the IRES. The level of expression of the second gene under control of the IRES can be variable; in the case of the encephalomyocarditis virus IRES, expression can range anywhere between 6 and 100% that of cap-dependent translation from the cytomegalovirus (CMV) promoter, depending on cell type and the gene in question (161).

Another method of dual gene synthesis is to have each gene under the control of its own promoter. However, the use of different promoters can result in protein expression levels that are also dependent on cell-type. Furthermore, promoter interference can occur, where the transcription from one promoter can interfere with that of the other (162, 163). Recently, Amendola *et al.* described the use of a synthetic bidirectional promoter for use in a lentiviral transfer vector

(164). The synthetic promoter consisted of an 'efficient' promoter, either the human ubiquitin C or phosphoglycerate kinase (PGK) promoters, joined in opposite orientation to a minimal core promoter derived from CMV. The upstream elements contained in the 'efficient' promoter, effectively flanked by two core promoters, could then drive transcription in both directions.

Project Rationale

A previous study from our laboratory described the pHERO DNA plasmid vaccine system (143). The plasmid backbone encodes genes promoting episomal stability allowing it to replicate in dividing cells. In this study, the pHERO plasmid, encoded M11L as well as gp140, a secretable form of HIV gp120 also containing the gp41 ectodomain. Inclusion of M11L in this vector resulted in decreased levels of HIV envelope glycoprotein-mediated apoptosis, as well as an increase HIV-specific $CD8^+$ T cell response (143). In conjunction with boosting with a canarypox virus encoding the HIV envelope glycoprotein, gag, and a portion of the protease gene, the effects were further increased. The presence of M11L also stimulated expansion of the central and effector memory $CD8^+$ T cell populations. In this study, the DNA vector was administered intramuscularly following cardiotoxin treatment. Cardiotoxin treatment caused local inflammation, promoting infiltration of inflammation responsive cells. It was postulated that the infiltrating cells, namely DCs, were responsible for uptake of the DNA vector, and subsequent synthesis of immunogenic peptides and presentation to Th1 cells in the context of MHC-I. Furthermore, as gp140 is a secretable form of HIV Env, it is possible that muscle cells that took up the

vaccine subsequently produced and secreted gp140. Regional APCs would then be able to take up the secreted protein and cross-present it to T cells in the lymph node while increases in longevity mediated by M11L may increase persistence within the lymph node. This would then lead to increased antigen presentation, and the stronger immune response.

In order to recapitulate this effect specifically in DCs, it is necessary to find an efficient method to transfer the appropriate genes into them. Furthermore, because coordinate expression of multiple genes is desired, the vector of choice must be multicistronic. In the case of a lentivirus vector, the two genes can be under the control of two promoters functioning bidirectionally, or a single promoter and an IRES between the genes. Different methods of both gene transfer and vector organization have to be assessed in order to determine the most effective way of obtaining suitable HIV gene expression and transfection/transduction efficiency.

Hypothesis and Objectives

Based on what has been shown about M11L and the previous findings concerning its use in the pHERO system, I hypothesize that expression of M11L in DCs results in increased DC longevity. To test my hypothesis, I have set a number of research objectives for this project. First, I will identify an effective method of gene transfer into DCs. By assessing a number of different gene transfer methods, I should arrive at one that provides sufficient gene transfer efficiency while also preserving cell viability. Secondly, I have to optimize

conditions coordinate dual-gene expression. Lastly, I will assess the effects of lentiviral transduction on DCs in culture from both a short-term and long-term perspective. Refinement of this system can demonstrate a novel method of boosting antigen presentation, especially in cases such as HIV where immunizing peptides may be cytotoxic. This can serve as a foundation for future studies of DC-based therapies.

Materials and Methods

Cell Culture

HEK293T (human embryonic kidney cells) and L cells (rat fibroblasts) were maintained in reconstituted powdered Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen) prepared according to manufacturer's instructions and supplemented with 10% FBS, 10mg/mL penicillin-streptomycin, and 200mM L-glutamine (complete DMEM).

DC2.4 (immature C57BL/6 mouse DCs) cells (165)were maintained in Roswell Park Memorial Institute (RPMI) 1640 culture medium supplemented with 10% fetal calf serum, non-essential amino acids, L-glutamine, sodium pyruvate, penicillin-streptomycin, and β-mercaptoethanol (complete RPMI 1640).

Transfection

Non-viral transfection methods were carried out in DC2.4 cells, an immortalized immature murine DC cell line (165). Transfection with Lipofectamine (Invitrogen) was carried out according to manufacturer's instructions. Briefly, 2×10^6 cells were placed into each well of a 6-well plate in 1.5mL of complete RPMI 1640. Plasmid (4 μg) and 10μL of Lipofectamine were each mixed into 250μL of serum free RPMI 1640. After 5 minutes of incubation, the diluted DNA and Lipofectamine were combined, and incubated for 20 minutes. The liposome-DNA complexes were then added dropwise to each well. After 24 hours, media was removed and replaced with fresh complete RPMI 1640. Analysis was performed 48 hours after transfection.

Transfection with Turbofect (Fermentas) was performed according to the manufacturer's instructions with some minor modifications. Twenty-four hours prior to transfection approximately 2 x 10^6 DC2.4 cells were plated in 4mL of complete RPMI 1640. At the time of transfection, 4µg of plasmid DNA was diluted in 400uL serum free RPMI 1640. Six microliters of Turbofect was then added to the diluted DNA sample, vortexed, and incubated at room temperature for 20 minutes. The DNA-polymer complexes were then added dropwise to the wells. Analysis was performed 48 hours after transfection.

Nucleofection

Nucleofection was performed according to manufacturer's instructions using the mouse DC Nucleofector kit (Lonza). Approximately 2.5 x 10^5 cells were resuspended in 100µL nucleofection solution. 2µg of DNA was then added to the cell suspension, and transferred to an Amaxa certified cuvette. The cells were transfected with the program Y-001for immature DCs. Pre-warmed (400µL) complete RPMI 1640 was added after Nucleofection to each cuvette. The transfected cell suspension was added to 400uL of pre-warmed complete RPMI 1640 in a 48 well plate. Analysis was performed 24 hours after Nucleofection.

Generation of Lentiviral Transfer Vectors

IRES containing vectors were based on the pCCL-rHER2/neu-IRES-M11L transfer vector provided by the laboratory of Dr. Jeffrey Medin at the University of Toronto. To generate the pCCL-EGFP vector, the rHER2/neu gene and the IRES-M11L fragment were excised using Asc I and Sal I-HF (New England

Biolabs), and purified the vector backbone by agarose gel electrophoresis followed by gel extraction using a QIAquick Gel Extraction Kit (QIAGEN). The enhanced green fluorescence protein (EGFP) gene was amplified from the pEGFP-N1 expression vector (Clonetech) using primers containing a 5' Asc I site and a 3'Sal I site (GAGGGCGCGCCATGGTGAGCAAGG and AGGGTCGACTTACTTGTACAGC). The PCR product was then ligated into the linearized pCCL vector backbone. The assembled plasmid was electroporated into Stbl-4 electrocompetent *E. coli* cells (Invitrogen), and correctness verified by restriction digest, PCR, and DNA sequencing.

To generate the pCCL-EGFP-IRES-M11L fragment, the IRES-M11L cassette was amplified by PCR from the original pCCL-rHER2/neu-IRES-M11L vector using primers containing a 5' and 3' Sal I site (TTCTTGTCGACGCCCCTCTCCCTCCCCCCCCC and TTCTTGTCGACGCGGCCGCCTAGGTCCCTCGGT). The pCCL-EGFP vector was linearized using Sal I and treated with calf intestinal alkaline phosphatase (Invitrogen) to prevent self-ligation. The treated linear plasmid was purified by phenol-chloroform extraction. The amplified IRES-M11L fragment was digested with Sal I and ligated into the dephosphorylated linear pCCL-EGFP vector immediately downstream of the EGFP coding region. The assembled plasmid was electroporated into Stbl-4 *E. coli* electrocompetent cells and verified by restriction digest, orientation specific PCR, and DNA sequencing.

Generation of the pCCL-EGFP-minCMV-hPGK-M11L vector began with the pCCL-EGFP-minCMV-hPGK-Luc bidirectional transfer vector supplied by the

laboratory of Dr. Jeffrey Medin. The M11L fragment was amplified from a purified IRES-M11L fragment using PCR with primers containing a 5'Pst I site and a 3'Sal I (AGGCCTGCAGATGATGTCTCGTTTAAAGACGG and GGTAGTCGACC TAGGTCCCTCGGTACCATTTT) site. The luciferase gene was excised from the starting plasmid using Pst I (Invitrogen) and Sal I-HF (New England Biolabs), and the vector backbone was purified by agarose gel electrophoresis followed by extraction using the PureLink Quick Gel Extraction Kit (Invitrogen). The M11L fragment was ligated into the linearized backbone and transformed into chemically competent DH5α *E. coli* cells (Invitrogen). The plasmids were verified by restriction digest and DNA sequencing.

Protein Extraction and Immunoblotting

Transfected or transduced cells were washed twice with ice-cold phosphate buffered saline (PBS) and lysed on the plate with RIPA buffer supplemented with 1 tablet of 50x complete protease inhibitor. After incubation on ice for 20 minutes, cell lysates were centrifuged at 16000 x g for 10 minutes at 4°C. Supernatants were transferred to new tubes, and protein concentration was quantified by Bio-Rad protein assay using an Ascent Multiskan (Thermo Scientific). Samples were stored at -80°C.

Soluble proteins (20μg) were subjected to SDS-PAGE using a 16% denaturing gel and transferred to a polyvinylidene difluoride FluoroTrans W membrane (Pall Scientific). Immunoblotting was performed using a 1:100 dilution of rabbit anti-M11L polyclonal antibody (134), and a 1:5000 dilution anti-GAPDH

antibody (Sigma), followed by 1:10000 dilutions of a goat-anti-rabbit or goat-anti-mouse secondary antibody conjugated to 800nm and 680nm infrared dyes respectively (LiCor Biosciences, donated by the laboratory of Dr. Stephen Barr, University of Western Ontario). Proteins were visualized using a LiCor Odyssey Infrared Imaging System.

Production of Lentiviral Vectors

The construct pMDG was used to generate the VSV-G viral envelope protein, while the *gag*, *pol*, and *rev* genes were expressed from pCMV. The transfer vectors used were the generated pCCL-EGFP, pCCL-EGFP-IRES-M11L, pCCL-EGFP-hPGK-minCMV-Luc and pCCL-EGFP-hPGK-minCMV-M11L.

In brief, 15cm^2 tissue culture treated dishes were seeded with approximately 15 x 10^6 low passage HEK293T cells 6 hours prior to transfection in complete DMEM. The envelope plasmid (pMDG; 3.8µg), packaging plasmid (19.01µg), and transfer plasmid (19.01µg) were suspended using 150mM NaCl to a volume of 1.125mL, and added 126nmol of polyethylenimine (Sigma Aldrich) dissolved in a total of 1.125mL. The transfection mixture was vortexed, added to the cells, and incubated overnight at 5% CO_2 and 37°C. The next morning, media was discarded and replaced with 15mL of fresh complete DMEM. At 25 hour intervals following the initial media change, culture supernatants were collected and replenished up to 3 times. Collected supernatants were filtered through a 0.45µm filter, and stored at 4°C prior to concentration.

To concentrate virus, 30mL of supernatant was subjected to ultracentrifugation for 2.5 hours at 4°C and 25 000rpm in an SW-28 rotor using a Beckman LM-8 ultracentrifuge. Pellets were resuspended in 100µL of Hank's buffered saline solution (HBSS) supplemented with 5% bovine serum albumin (BSA; HBSS/BSA). Pellets were pooled, re-aliquoted in 100µL volumes, and stored at -80°C. To avoid repetitive freeze-thaw cycles, all thawed vials were subsequently stored at 4°C for further use.

To titer concentrated and unconcentrated virus, approximately 8 x 10^5 HEK293T cells were seeded onto 6-well plates at least 4 hours prior to transduction. At the time of transduction, media was removed and replaced with 1mL of media containing serial 10-fold dilutions of virus and 8µg/mL of protamine sulfate. Following overnight incubation, media was removed and replaced with 4mL of complete DMEM. After an additional 24 hours, virus was titered by counting of EGFP-positive foci by fluorescent microscopy.

Cell Transduction

Transduction of primary bone marrow derived DCs (BMDCs) was performed immediately following DC enrichment. Two million BMDCs were added to each well of a 6 well plate. Lentivirus was added at a multiplicity of infection (MOI) of 5, supplemented 10µg/mL with DEAE-dextran, in a culture volume of 1mL of complete RPMI plus 4ng/mL IL-4 and 1000U/mL GM-CSF. At 16 hours after infection, 3mL of complete RPMI plus IL-4 and GM-CSF was added to cells.

Transduction of L cells was performed by adding 2 x 10^6 cells to each well of a 6 well plate. Cells were given at least 6 hours to adhere to the plate. Media was then removed and virus was added at an MOI of 5 supplemented with 8µg/mL of protamine sulfate (Sigma Aldrich) and complete DMEM to a total culture volume of 1mL. At 16 hours post infection, media was removed and replaced with 2mL of fresh DMEM.

Induction of Apoptosis

L cells were transduced with pCCL-EGFP or pCCL-EGFP-IRES-M11L at an MOI of 5. After 48 hours, cultures were treated with staurosporine (STS; Invitrogen) dissolved in dimethyl sulfoxide (Bioshop; DMSO) at a final concentration of 4µM for 8 hours. Following the treatment time, supernatants and floating cells were retained, and attached cells were washed with PBS and lifted using 0.25% trypsin-EDTA. All cells were washed 2 times with PBS, once with 1X binding buffer (BD Pharmingen) and resuspended to make 100µL aliquots in flow cytometry tubes. Levels of apoptosis were assessed via staining for 7-aminoactinomcin D (7-AAD; 1:20, BD Pharmingen) and annexin V-PE (1:20; BD Pharmingen). Excess stain was washed once and resuspended in 400µL of 1X binding buffer. Stained cells were run through a BD FACS Calibur and analyzed using FlowJo software (TreeStar).

Mouse Bone Marrow DC Isolation and Culture

Mice were used in accordance with protocols approved by the University of Western Ontario Animal Care and Use Subcommittee. Bone marrow was

isolated from femurs and tibias of 6 to 8-week old C57Bl/6 mice as previously described (166). Briefly, bone marrow cells were cultured for 4 days in complete RPMI supplemented with the 1000 U/mL of GM-CSF and 4ng/mL of IL-4 (donated by Schering-Plough via Dr. Peta J. O'Connell). At day 4, dendritic cells were enriched by centrifugation over a 13.5% histodenz gradient (25 min, 500 x g).

Cell Staining and Flow Cytometry

Cells were prepared for flow cytometry by washing approximately 2 x 10^6 cells in a 6-well plate with ice cold HBSS supplemented with 1% BSA. Lightly attached cells were removed by gentle pipetting. Cells were then washed 3 times with HBSS+BSA and 5 minute 500 x g centrifugations. Cell pellets were resuspended in 1mL HBSS/BSA and blocked with 50µL normal goat serum. Cells were then washed, and resuspended to make 100µL aliquots into flow cytometry tubes. Cells were then stained with CD86-PerCP (1:400; BioLegend), CD11c-PE (1:600; BioLegend), and Far Red Live/Dead Viability dye (1:4500; Invitrogen). Excess antibody was washed, and pelletted cells were resuspended in 300µL HBSS, and fixed with an additional 100µL of 4% paraformaldehyde dissolved in PBS. Stained cells were run through a BD FACS Calibur and analyzed with FlowJo Software (TreeStar).

Statistics

GraphPad Prism 4 software was used to perform one way analysis of variance (ANOVA) testing with Tukey's post test when comparing 3 or more

groups, two way ANOVA testing with Bonferroni post test in two variable experiments, and student's T test when comparing 2 groups. Data are presented as group means with ± standard error. Unless otherwise stated, data are representative of n=3 where n represents the number of independent experiments performed. A P-value <0.05 was deemed to be statistically significant.

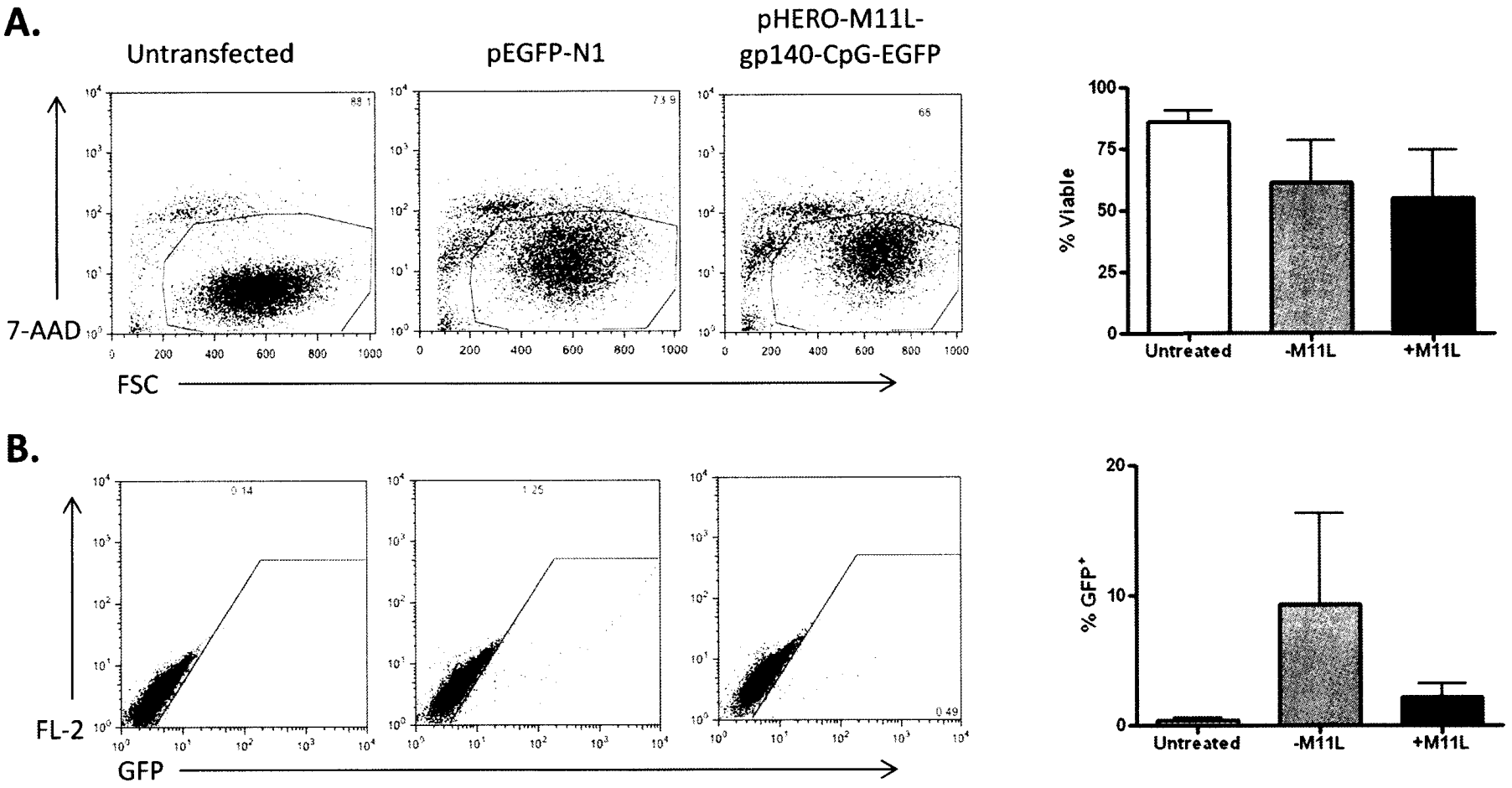
Results

Non-viral Transfection Methods Are Not Effective in DCs

To asses the efficiency of gene transfer into DCs, a number of non-viral vector-mediated transfection methods were attempted. To perform these studies, I used DC2.4 cells, an immortalized line of immature murine dendritic cells (165). I tested three of the more common transfection methods: cationic polymer transfection, liposome mediated transfection, and nucleofection. In order to obtain both gp140 and M11L expression, I used the previously characterized pHERO-M11L-gp140-CpG-EGFP (143) in conjunction with a control EGFP expression vector, pEGFP-N1. In the case of nucleofection, the control vector used was the manufacturer's supplied pMAX-GFP expression vector. EGFP expression was determined by flow cytometry, and viability was assessed by staining with 7-AAD.

Cationic polymer-mediated transfection was performed on DC2.4 cells using Turbofect, a new transfection reagent marketed by Fermentas (Fig. 1). The reagent control resulted in a viability of 86.1±4.3%. Viability following transfection with the control pEGFP-N1 was seen to be 61.8±16.8%. When the pHERO-M11L-gp140-CpG-EGFP vector was used, viability dropped further to 55.2±19.4%. Statistical analysis proved these differences to not be significant. However, there did appear to be a slight trend towards a lower viability upon transfection of the expression plasmids. To determine transfection efficiency, I gated on viable 7-AAD$^-$ cells and the sizes of the EGFP$^+$ populations were

Figure 1 – Cationic polymer mediated transfection does not result in significant levels of pHERO transfection in DC2.4 cells. DC2.4 cells were transfected with pEGFP-N1 or pHERO-M11L-gp140-CpG-EGFP using the cationic polymer solution, Turbofect. Forty-eight hours following transfection, cells were collected and analyzed for viability and GFP expression by flow cytometry. (A) Representative flow cytometric density plots showing viable (7-AAD$^-$ cells) and pooled data from 3 independent experiments showing the mean ± standard error. (B) Representative flow cytometric plots showing GFP expressing cells as a percentage of viable cells as gated in *A*, and pooled data from 3 independent experiments. One way ANOVA testing was performed to determine any significant differences between groups.


A.
Untransfected
pEGFP-N1
pHERO-M11L-
gp140-CpG-EGFP
7-AAD
FSC
88.1
73.9
65
% Viable
100
75
50
25
0
Untreated
-M11L
+M11L
B.
FL-2
GFP
0.14
1.25
0.49
% GFP+
20
10
0
Untreated
-M11L
+M11L

assessed. More transfectants were observed when using the pEGFP-N1 control vector, with approximately 9.4±7.0% of viable cells being transfected. However, use of the pHERO-M11L-gp140-CpG-EGFP vector resulted in a lower, though statistically insignificant, level of transfection, as only 2.2±1.0% of viable cells expressed EGFP as determined by one way ANOVA.

Liposome mediated transfection was performed in DC2.4 cells using the commercially available reagent Lipofectamine 2000 from Invitrogen (Fig 2). This reagent is among the most common used for transfection, normally capable of transfecting a wide variety of cell lines. As in the case of the Turbofect reagent, treatment with Lipofectamine alone did not result in a large loss in viability (93.5±1.7%). When the pEGFP-N1 vector was used in transfection, viability decreased significantly to 77.3±5.5%. The drop in viability was more evident when transfecting the pHERO-M11L-gp140-CpG-EGFP vector, as viability decreased to 73.4±4.1%. I then examined the percentage of viable cells that also expressed EGFP. As I observed with the Turbofect reagent, the pEGFP-N1 vector was transfected at a slightly higher efficiency than pHERO-M11L-gp140-CpG-EGFP, 7.5±3.8% compared to 2.4±1.4%. However, this difference was not observed to be statistically significant as determined by one way ANOVA.

I also attempted to use Nucleofection developed by Amaxa due to its reputation of being able to transfect "hard-to-transfect" cells (Fig.3). However, it is important to note that Nucleofection utilizes the smallest number of starting cells (2.5×10^5). As a transfection control, I utilized the pMAX-GFP expression vector supplied by Amaxa. Nucleofection of DC2.4 cells in the absence of plasmid was

Figure 2 – Liposome mediated transfection does not result in significant levels of pHERO transfection in DC2.4 cells. DC2.4 cells were transfected with pEGFP-N1 or pHERO-M11L-gp140-CpG-EGFP using the liposome solution, Lipofectamine 2000. Forty-eight hours following transfection, cells were collected and analyzed for viability and GFP expression by flow cytometry. (A) Representative flow cytometric density plots showing viable (7-AAD^- cells) and pooled data from 3 independent experiments showing the mean ± standard error. (B) Representative flow cytometric plots showing GFP expressing cells as a percentage of viable cells as gated in *A*, and pooled data from 3 independent experiments. One way ANOVA testing was performed to determine any significant differences between groups. $^*P < 0.05$.

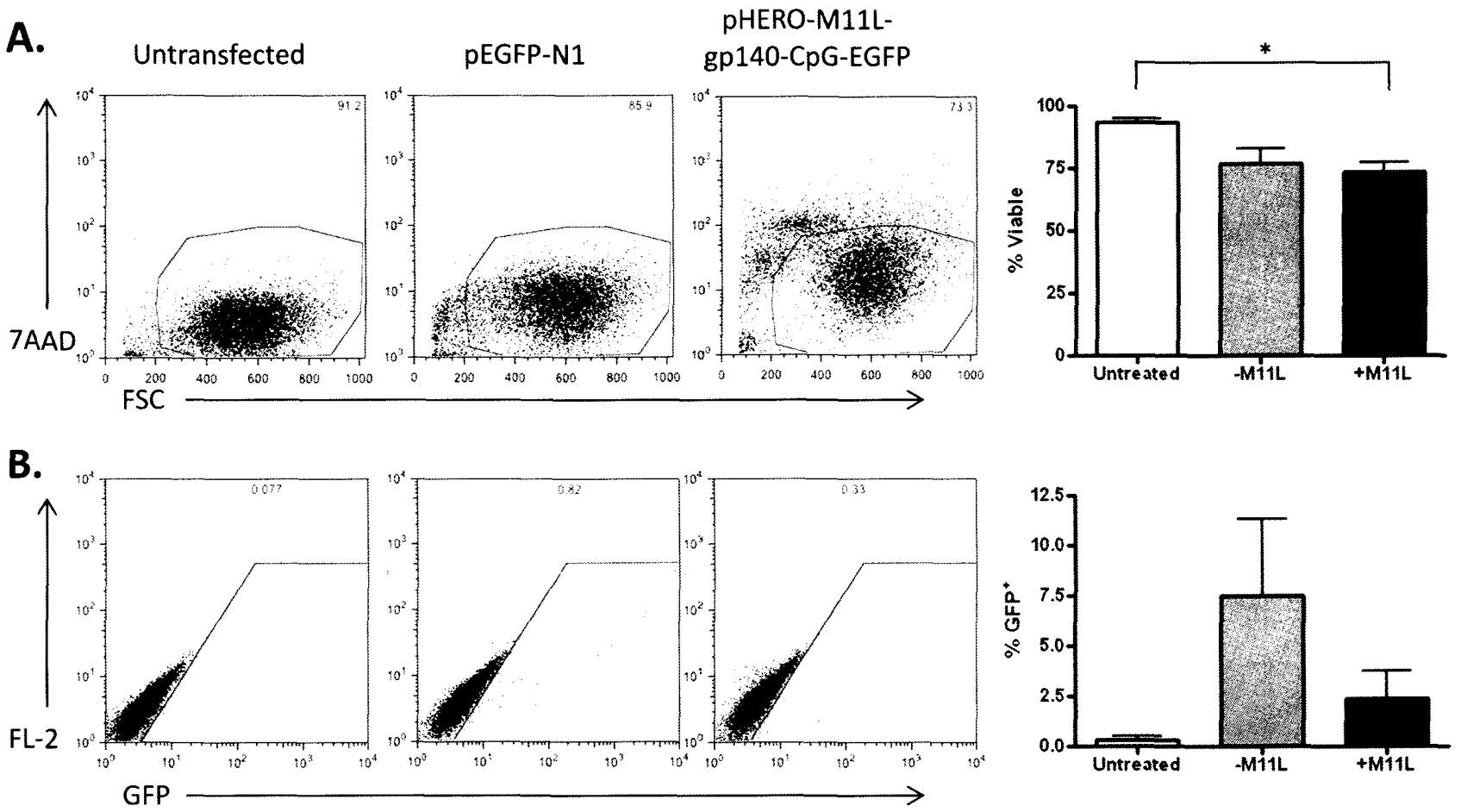
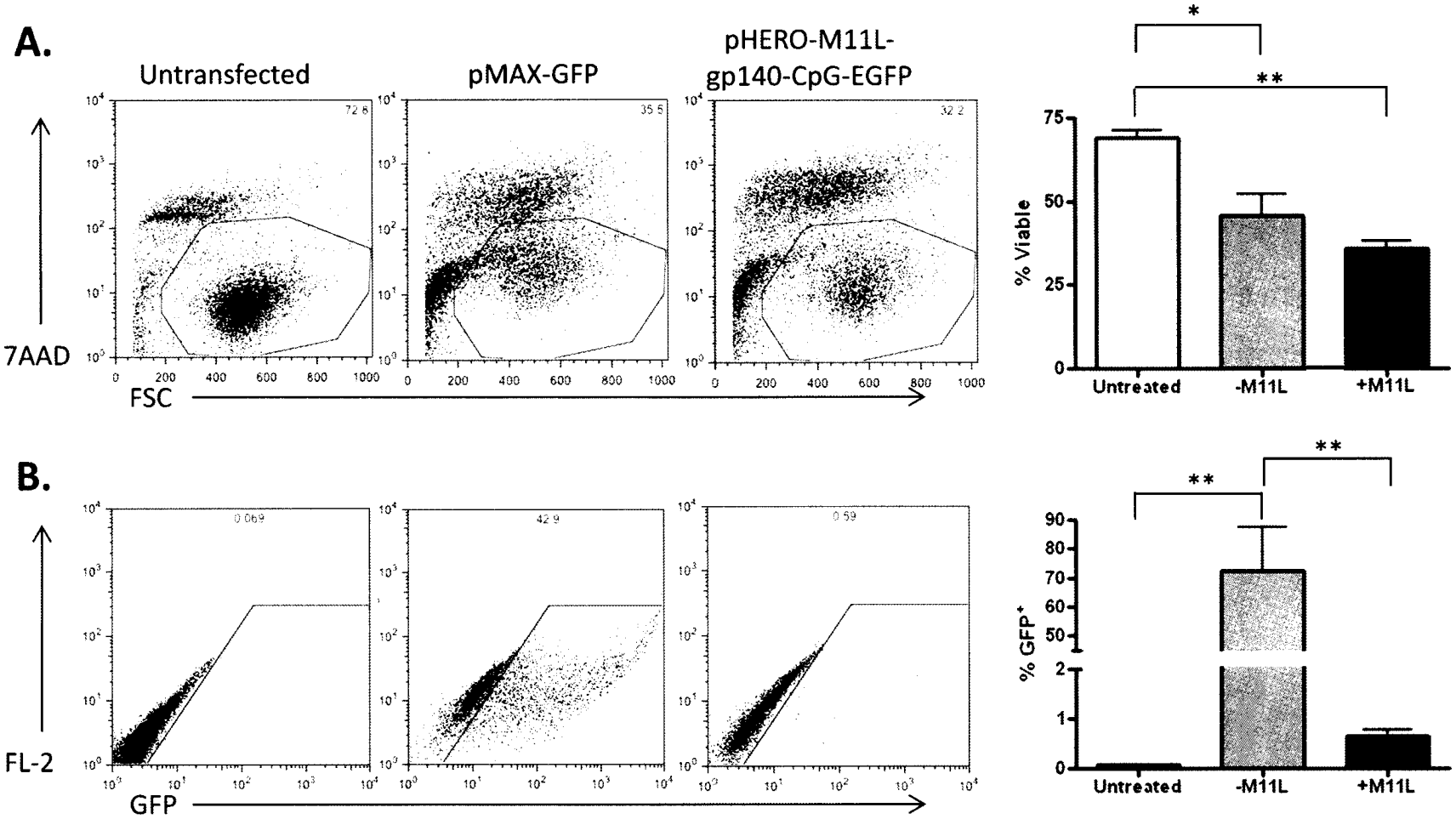

A.
Untransfected
pEGFP-N1
pHERO-M11L-
gp140-CpG-EGFP
7AAD
FSC
91.2
85.9
73.3
*
% Viable
100
75
50
25
0
Untreated
-M11L
+M11L
B.
FL-2
GFP
0.077
0.82
0.33
% GFP+
12.5
10.0
7.5
5.0
2.5
0.0
Untreated
-M11L
+M11L

Figure 3 – Nucleofection is not effective in DC2.4 cells. DC2.4 cells were transfected with pMAX-GFP or pHERO-M11L-gp140-CpG-EGFP via Nucleofection. Twenty-four hours following transfection, cells were collected and analyzed for viability and GFP expression by flow cytometry. (A) Representative flow cytometric density plots showing viable (7-AAD$^-$ cells) and pooled data from 3 independent experiments showing the mean ± standard error. (B) Representative flow cytometric plots showing GFP expressing cells as a percentage of viable cells as gated in *A*, and pooled data from 3 independent experiments. One way ANOVA testing was performed to determine any significant differences between groups. *P < 0.05, **P < 0.01


A.
Untransfected
pMAX-GFP
pHERO-M11L-
gp140-CpG-EGFP
7AAD
FSC
72.8
35.5
32.2
*
**
% Viable
75
50
25
0
Untreated
-M11L
+M11L
B.
FL-2
GFP
0.069
42.9
0.59
**
**
% GFP+
90
80
70
60
50
2
1
0
Untreated
-M11L
+M11L

shown to result in 69.2±2.3% viability, suggesting that the pulsing is more cytotoxic than both Lipofectamine and Turbofect alone. Inclusion of any of the plasmids significantly reduced viability, as nucleofection of pMAX-GFP resulted in 46±6.1% viability, and 35.8±2.1% when using pHERO-M11L-gp140-CpG-EGFP. Nucleofection of the GFP control plasmid occurred at an efficiency of 72.5±14.9%. However, there was a large decrease in transfection efficiency when using the pHERO-M11L-gp140-CpG-EGFP, as only 0.6±0.1% of viable cells expressed EGFP.

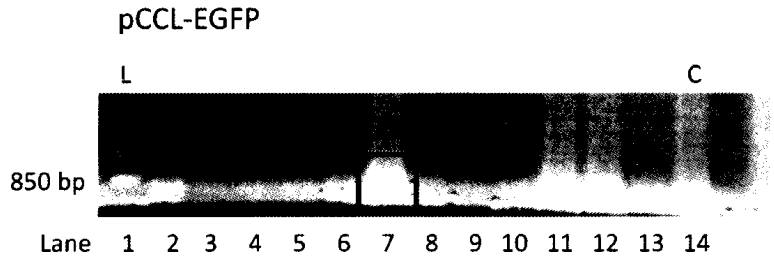
Generation of Lentivirus Transfer Vector

In addition to non-viral methods of gene transfer, I also attempted to use lentiviral vectors to transduce the DCs. This method necessitated the construction of transfer vectors encoding the integration cassette to be included into the virus particle.

IRES-containing vectors were constructed starting from the pCCL-rHer2/neu-IRES-M11L vector obtained from Dr. Jeffrey Medin's laboratory (University of Toronto). The integration cassette of this vector contained rHer2/neu downstream of the elongation factor 1 alpha (EF1α) promoter, flanked by a 5' Asc I site and a 3' Sal I site, and an IRES-M11L fragment immediately downstream of rHer2/neu, flanked at both 5' and 3' ends with an Asc I site. I excised both fragments by Asc I/Sal I double digest, and amplified the EGFP gene from the pEGFP-N1 vector, including a 5' Asc I site and 3' Sal I site. The EGFP fragment was ligated to the pCCL backbone. PCR amplification of the

Figure 4 – Generation of pCCL-EGFP lentiviral transfer vector. The pCCL backbone was obtained by digesting a pCCL-rHer2/neu-IRES-M11L lentiviral transfer vector. EGFP was ligated downstream of the EF1α promoter. (A) Agarose gel electrophoresis of PCR amplification products using EGFP-specific primers. Red square denotes clone used in sequencing. L represents DNA size ladder. C represents control pEGFP-N1 vector. (B) Sequence comparison of selected clone and EF1α sequences.

A.



B.

```
>EF1alpha       GTGCCGTGTGTGGTTCCCGCGGGCCTGGCCTCTTTACGGGTTATGGCCCTTGCGTGCCTT 300
>pCCL-EGFP      GTNCCGNNNGTNGT-CCCGCGNNC-TGGCNTCNTTNNGGNTNN--GGCCCTNCGTNCCT- 70
                ** ***   ** ** ******  * **** ** **  ** *    * ** * *** ***

>EF1alpha       GAATTACTTCCACCTGGCTGCAGTACGTGATTCTTGATCCCGAGCTTCGGGTTGGAAGTG 360
>pCCL-EGFP      GAATTACTNCCNCCNNGCTGCAGTACGTGATTNTNNATCCCGAGCTTCGGGTTGGAAGTG 130
                ******** ** **  ********** * ************************

>EF1alpha       GGTGGGAGAGTTCGAGGCCTTGCGCTTAAGGAGCCCCTTCGCCTCGTGCTTGAGTTGAGG 420
>pCCL-EGFP      GGTGGGAGAGTTCGAGGCCTTGCGCTTAAGGAGCCCCTTCGCCTCGTGCTTGAGTNGAGG 190
                ************************************************** ****

>EF1alpha       CCTGGCCTGGGCGCTGGGGCCGCCGCGTGCGAATCTGGTGGCACCTTCGCGCCTGTCTCG 480
>pCCL-EGFP      CCTGGCCTGGGCGCTGGGGCCGCCGCGTGCGAATCTGGTGGCNCCTTCGCGCCTGTCTCG 250
                ****************************************** ****************

>EF1alpha       CTGCTTTCGATAAGTCTCTAGCCATTTAAAATTTTTGATGACCTGCTGCGACGCTTTTTT 540
>pCCL-EGFP      CTGCTTTCGATAAGTCTCTAGCCATTTAAAATTTTTGATGNCCTGCTGCGACGCTTTTTT 310
                ****************************************** ****************

>EF1alpha       TCTGGCAAGATAGTCTTGTAAATGCGGGCCAAGATCTGCACACTGGTATTTCGGTTTTTG 600
>pCCL-EGFP      TCTGGCAAGATAGTCTTGTAAATGCGGGCCAAGATCTGCACACTGGTATTTCGGTTTTTG 370
                ************************************************************

>EF1alpha       GGGCCGCGGGCGGCGACGGGGCCCGTGCGTCCCAGCGCACATGTTCGGCGAGGCGGGGCC 660
>pCCL-EGFP      GGGCCGCGGGCGGCGACGGGGCCCGTGCGTCCCAGCGCACATGTTCGGCGAGGCGGGGCC 430
                ************************************************************

>EF1alpha       TGCGAGCGCGGCCACCGAGAATCGGACGGGGGTAGTCTCAAGCTGGCCGGCCTGCTCTGG 720
>pCCL-EGFP      TGCGAGCGCGGCCACCGAGAATCGGACGGGGGTAGTCTCAAGCTGGCCGGCCTGCTCTGG 490
                ************************************************************

>EF1alpha       TGCCTGGCCTCGCGCCGCCGTGTATCGCCCCGCCCTGGGCGGCAAGGCTGGCCCGGTCGG 780
>pCCL-EGFP      TGCCTGGCCTCGCGCCGCCGTGTATCGCCCCGCCCTGGGCGGCAAGGCTGGCCCGGTCGG 550
                ************************************************************
```

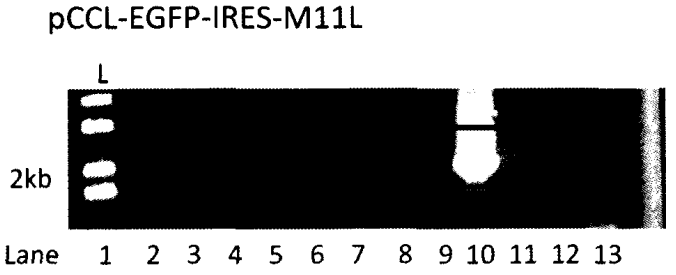
EGFP insert revealed an 850bp fragment in lanes 7, 11, 12, and 13, consistent with the size of gene (Fig. 4A). Further verification of the promoter was done by sequence alignment of the sample in lane 7 with the consensus sequence of the EF1α promoter (Fig 4B). Although gaps in the sequence were observed, the presence of the correct nucleotides was verified using alternate primers.

To generate pCCL-EGFP-IRES-M11L, the obtained pCCL-EGFP vector was linearized using Sal I and treated with calf alkaline phosphatase to prevent self-ligation. The IRES-M11L fragment was generated by PCR amplification containing Sal I sites at both 5' and 3' ends. After ligation, presence of the insert was determined by PCR amplification using primers at the 3' end of EGFP and the 3' end of M11L (Fig. 5A). As expected, I observed a band at a size of approximately 2kb in lane 10. DNA sequencing was performed and that of the generated vector was aligned with the sequence of the IRES-M11L fragment (Fig. 5B). Errors were observed in the IRES sequence, possibly influencing levels of M11L expression.. However, misread nucleotides and sequencing gaps may also be a product of the complex secondary structure inherent to the IRES region. The cloned M11L was, however, seen to match the consensus sequence (Accession number: M93049).

In addition to the IRES containing vectors, I generated a bicistronic transfer vector (pCCL-EGFP-minCMV-hPGK-M11L) utilizing a bidirectional promoter. We amplified the M11L gene from a previously purified IRES-M11L fragment adding a 5' Pst I site and 3' Sal I site. Starting from a pCCL-EGFP-minCMV-hPGK-Luc vector, the luciferase gene was excised using Pst I and Sal I.

Figure 5 – Generation of pCCL-EGFP-IRES-M11L lentiviral transfer vector. The IRES-M11L fragment was amplified by PCR and ligated into pCCL-EGFP immediately downstream of EGFP. (A) Agarose gel electrophoresis PCR amplified IRES-M11L using orientation specific primers. L corresponds to DNA size ladder. Red box denotes clone selected for sequencing. (B) Sequence comparison of IRES-M11L sequence with the selected pCCL-EGFP-IRES-M11L clone.

A.



B.

```
>IRES-M11L                  ----------------------------------------GTCGACGCCCCTCTCCCT 18
>pCCL-EGFP-IRES-M11L        NNNNNNNNNNNNNNNNNNNNNNNNTGNNNGANCTGTACANGTAAGTCGACGCCCCTCTCCCT 60
                                                                    ********************

>IRES-M11L                  CCCCCCCCCCCTAACGTTACTGGCCGAAGCCGCTTGGAATAAGGCCGGTGTGCGTTTGTCT 78
>pCCL-EGFP-IRES-M11L        CCCCCCC--CTAACGTTACTGGCCGAAGCCGCTTGGAATAAGGCCGGTGTGCGTTTGTCT 118
                            *******  **************************************************

>IRES-M11L                  ATATGTTATTTTCCACCATATTGCCGTCTTTTGGCAATGTGAGGGCCCGGAAACCTGGCC 138
>pCCL-EGFP-IRES-M11L        ATATGTTGTTTTCCACCATATTGCCGTCTTTTGGCAATGTGAGGGCCCGGAAACCTGGCC 178
                            ******* ****************************************************

>IRES-M11L                  CTGTCTTCTTGACGAGCATTCCTAGGGGTCTTTCCCCTCTCGCCAAAGGAATGCAAGGTC 198
>pCCL-EGFP-IRES-M11L        CTGTCTTCTTGACGAGCATTCCTAGGGGTCTTTCCCCTCTCGCCAAAGGAATGCAAGGTC 238
                            ************************************************************

>IRES-M11L                  TGTTGAATGTCGTGAAGGAAGCAGTTCCTCTGGAAGCTTCTTGAAGACAAACAACGTCTG 258
>pCCL-EGFP-IRES-M11L        TGTTGAATGTCGTGAAGGAAGCAGTTCCTCTGGAAGCTTCTTGAAGACAAACAACGTCTG 298
                            ************************************************************

>IRES-M11L                  TAGCGACCCTTTGCAGGCAGCGGAACCCCCCACCTGGCGACAGGTGCCTCTGCGGCCAAA 318
>pCCL-EGFP-IRES-M11L        TAGCGACCCTTTGCAGGCAGCGGAACCCCTCACCTGGCGACAGGTGCCTCTGCGGCCAAA 358
                            ***************************** ******************************

>IRES-M11L                  AGCCACGTGTATAAGATACACCTGCAAAGGCGGCACAACCCCAGTGCCACGTTGTGAGTT 378
>pCCL-EGFP-IRES-M11L        AGCCACGTGTATAAGATACACCTGCAAAGGCGGCACAACCCCAGTGCCACGTTGTGAGTT 418
                            ************************************************************

>IRES-M11L                  GGATAGTTGTGGAAAGAGTCAAATGGCTCTCCTCAAGCGTATTCAACAAGGGGCTGAAGG 438
>pCCL-EGFP-IRES-M11L        GGATAGTTGTGGAAAGAGTCAAATGGCTCTCCTCAAGCGTATTCAACAAGGGGCTGAAGG 478
                            ************************************************************

>IRES-M11L                  ATGCCCAGAAGGTACCCCATTGTATGGGATCTGATCTGGGGCCTCGGTACACATGCTTTA 498
>pCCL-EGFP-IRES-M11L        ATGCCCAGAAGGTACCCCATTGTATGGGATCTGATCTGGGGCCTCGGTACACATGCTTTA 538
                            ************************************************************

>IRES-M11L                  CATGTGTTTAGTCGAGGTTAAAAAAACGTCTAGGCCCCCCGAACCACGGGGACGTGGTTT 558
>pCCL-EGFP-IRES-M11L        CATGTGTTTAGTCGAGGTTAAAAAAACGTCTAGGCCCCCCGAACCACGGGGACGTGGTTT 598
                            ************************************************************
```

I then inserted the M11L fragment, whose presence was verified using M11L specific primers. The insert was verified by DNA sequencing and compared to the consensus EGFP sequence (Fig. 6; accession number U55762).

To show that both genes were being expressed, I transduced HEK293T cells with each transfer vector. EGFP expression was assessed by flow cytometry, while M11L expression was detected by western blotting (Fig. 7). As expected, I observed EGFP expression in all of the vectors examined (Fig. 7A). The pCCL-EGFP vector was observed to have the highest MFI of all vectors, 126.1±53.7. Furthermore, the level of EGFP expression was lower but not significantly when transducing with pCCL-EGFP-IRES-M11L, which had an MFI of 40.3. Interestingly, the pCCL-EGFP-minCMV-hPGK-Luc vector displayed an MFI of 106.5, similar to that of pCCL-EGFP. However, it did trend towards a lower difference with its companion pCCL-EGFP-minCMV-hPGK-M11L than with the IRES containing vectors (Fig. 7A top graph). The high variability in the level of EGFP expression may be explained by the use of different virus stocks in each attempt. When assessing M11L expression, I included the pHERO-M11L-CpG-gp140-EGFP vector as a control, which was shown previously to express M11L (143). Both the pCCL-EGFP-IRES-M11L and pCCL-EGFP-minCMV-hPGK-M11L vectors expressed M11L (Fig. 7B).

Effect of M11L On Staurosporine-induced Apoptosis

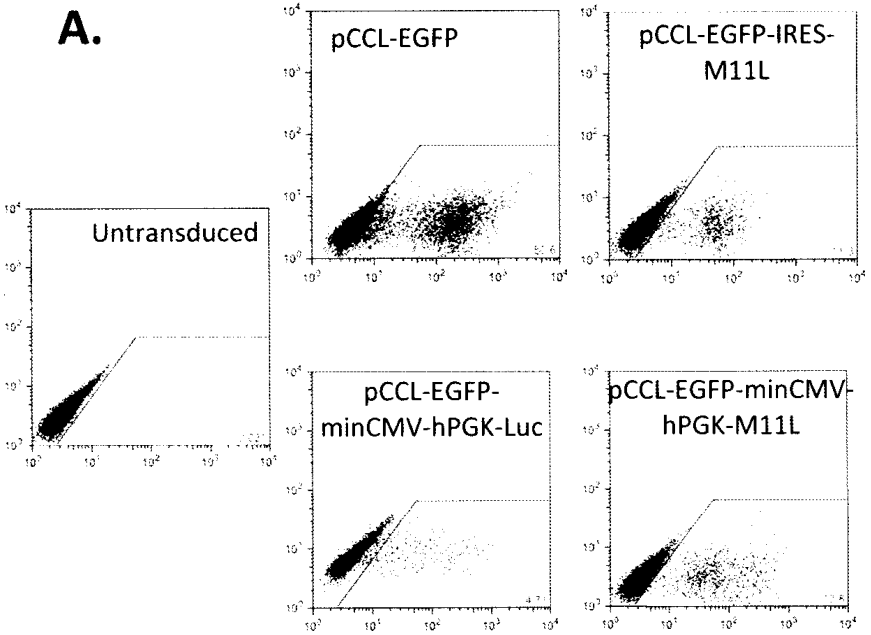
I next wanted to examine if M11L transduction resulted in protection from apoptosis. As rat L cells were previously used in the characterization of the anti-

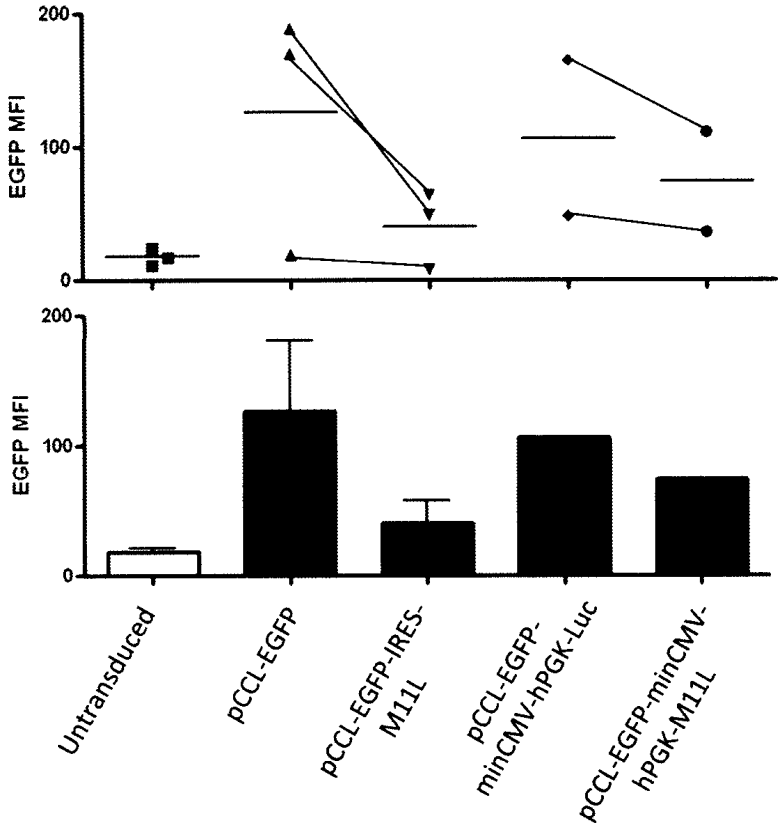
Figure 6 – Sequence analysis of pCCL-EGFP-minCMV-hPGK-M11L lentiviral transfer vector. The M11L gene was inserted downstream of the hPGK promoter. Sequencing was performed using an outward N-terminus EGFP sequencing primer. Shown is the alignment of the hPGK promoter controlling M11L expression, and the M11L gene.

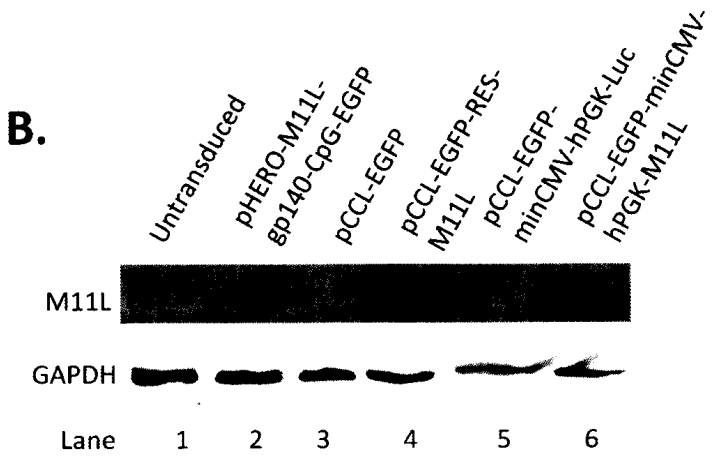
pCCL-EGFP-minhCMV-hPGK-M11L

```
>M11L                          ----------------------ATGATGTCTCGTTTAAAGACGGCCGTATA 29
>pCCL-EGFP-minCMV-hPGK-M11L    GGGGGATCCCCCGGGCTGCAGATGATGTCTCGTTTAAAGACGGCCGTATA 550
                                                      ****************************

>M11L                          CGATTATCTGAACGACGTGGATATAACGGAGTGTACGGAAATGGATCTAC 79
>pCCL-EGFP-minCMV-hPGK-M11L    CGATTATCTGAACGACGTGGATATAACGGAGTGTACGGAAATGGATCTAC 600
                               **************************************************

>M11L                          TGTGTCAGTTGAGTAATTGTTGCGATTTTATCAACGAAACGTACGCAAAA 129
>pCCL-EGFP-minCMV-hPGK-M11L    TGTGTCAGTTGAGTAATTGTTGCGATTTTATCAACGAAACGTACGCAAAA 650
                               **************************************************

>M11L                          AACTACGACACGTTGTATGATATCATGGAACGGGACATTTTGTCGTATAA 179
>pCCL-EGFP-minCMV-hPGK-M11L    AACTACGACACGTTGTATGATATCATGGAACGGGACATTTTGTCGTATAA 700
                               **************************************************

>M11L                          TATCGTGAACATTAAAAATACGTTGACGTTCGCGTTACGAGACGCGTCAC 229
>pCCL-EGFP-minCMV-hPGK-M11L    TATCGTGAACATTAAAAATACGTTGACGTTCGCGTTACGAGACGCGTCAC 750
                               **************************************************

>hPGK                          CCGGTAGGCGCCAACCGGCTCCGTTCTTTGGTGGCCCCTTCGCGCCACCT 50
>pCCL-EGFP-minCMV-hPGK-M11L    CCGGTAGGCGCCAACCGGCTCCGTTCTTTGGTGGCCCCTTCGCGCCACCT 50
                               **************************************************

>hPGK                          TCTACTCCTCCCCTAGTCAGGAAGTTCCCCCCCGCCCCGCAGCTCGCGTC 100
>pCCL-EGFP-minCMV-hPGK-M11L    TCTACTCCTCCCCTAGTCAGGAAGTTCCCCCCCGCCCCGCAGCTCGCGTC 100
                               **************************************************

>hPGK                          GTGCAGGACGTGACAAATGGAAGTAGCACGTCTCACTAGTCTCGTGCAGA 150
>pCCL-EGFP-minCMV-hPGK-M11L    GTGCAGGACGTGACAAATGGAAGTAGCACGTCTCACTAGTCTCGTGCAGA 150
                               **************************************************

>hPGK                          TGGACAGCACCGCTGAGCAATGGAAGCGGGTAGGCCTTTGGGGCAGCGGC 200
>pCCL-EGFP-minCMV-hPGK-M11L    TGGACAGCACCGCTGAGCAATGGAAGCGGGTAGGCCTTTGGGGCAGCGGC 200
                               **************************************************

>hPGK                          CAATAGCAGCTTTGCTCCTTCGCTTTCTGGGCTCAGAGGCTGGGAAGGGG 250
>pCCL-EGFP-minCMV-hPGK-M11L    CAATAGCAGCTTTGCTCCTTCGCTTTCTGGGCTCAGAGGCTGGGAAGGGG 250
                               **************************************************

>hPGK                          TGGGTCCGGGGGCGGGCTCAGGGGCGGGCTCAGGGGCGGGGCGGGCGCCC 300
>pCCL-EGFP-minCMV-hPGK-M11L    TGGGTCCGGGGGCGGGCTCAGGGGCGGGCTCAGGGGCGGGGCGGGCGCCC 300
                               **************************************************

>hPGK                          GAAGGTCCTCCGGAGGCCCGGCATTCTGCACGCTTCAAAAGCGCACGTCT 350
>pCCL-EGFP-minCMV-hPGK-M11L    GAAGGTCCTCCGGAGGCCCGGCATTCTGCACGCTTCAAAAGCGCACGTCT 350
                               **************************************************

>hPGK                          GCCGCGCTGTTCTCCTCTTCCTCATCTCCGGGCCTTTCGACCTGCAGCC 399
>pCCL-EGFP-minCMV-hPGK-M11L    GCCGCGCTGTTCTCCTCTTCCTCATCTCCGGGCCTTTCGACCTGCAGCC 399
                               **************************************************
```

Figure 7 – Protein expression in transduced HEK 293T cells. HEK 293T cells were transduced with the designated lentiviral vector at an MOI of 5 and analyzed 48 hours later. (A) Representative data showing the level of EGFP transduction and intensity in cells transduced with the designated viral vector. Upper graph compares EGFP expression in paired experiments in transduced cells. Horizontal bar represents mean of data. Bottom graph shows pooled data representative of n=3 (pCCL-EGFP and pCCL-EGFP-IRES-M11L) or n=2 (pCCL-EGFP-minCMV-hPGK-Luc and pCCL-EGFP-minCMV-hPGK-M11L) independent experiments. Student's t test was used to compare pCCL-EGFP with pCCL-EGFP-IRES-M11L and pCCL-EGFP-minCMV-hPGK-Luc with pCCL-EGFP-minCMV-hPGK-M11L. (B) Western blot showing M11L expression in transduced cells.


A.
Untransduced
pCCL-EGFP
pCCL-EGFP-IRES-M11L
pCCL-EGFP-minCMV-hPGK-Luc
pCCL-EGFP-minCMV-hPGK-M11L

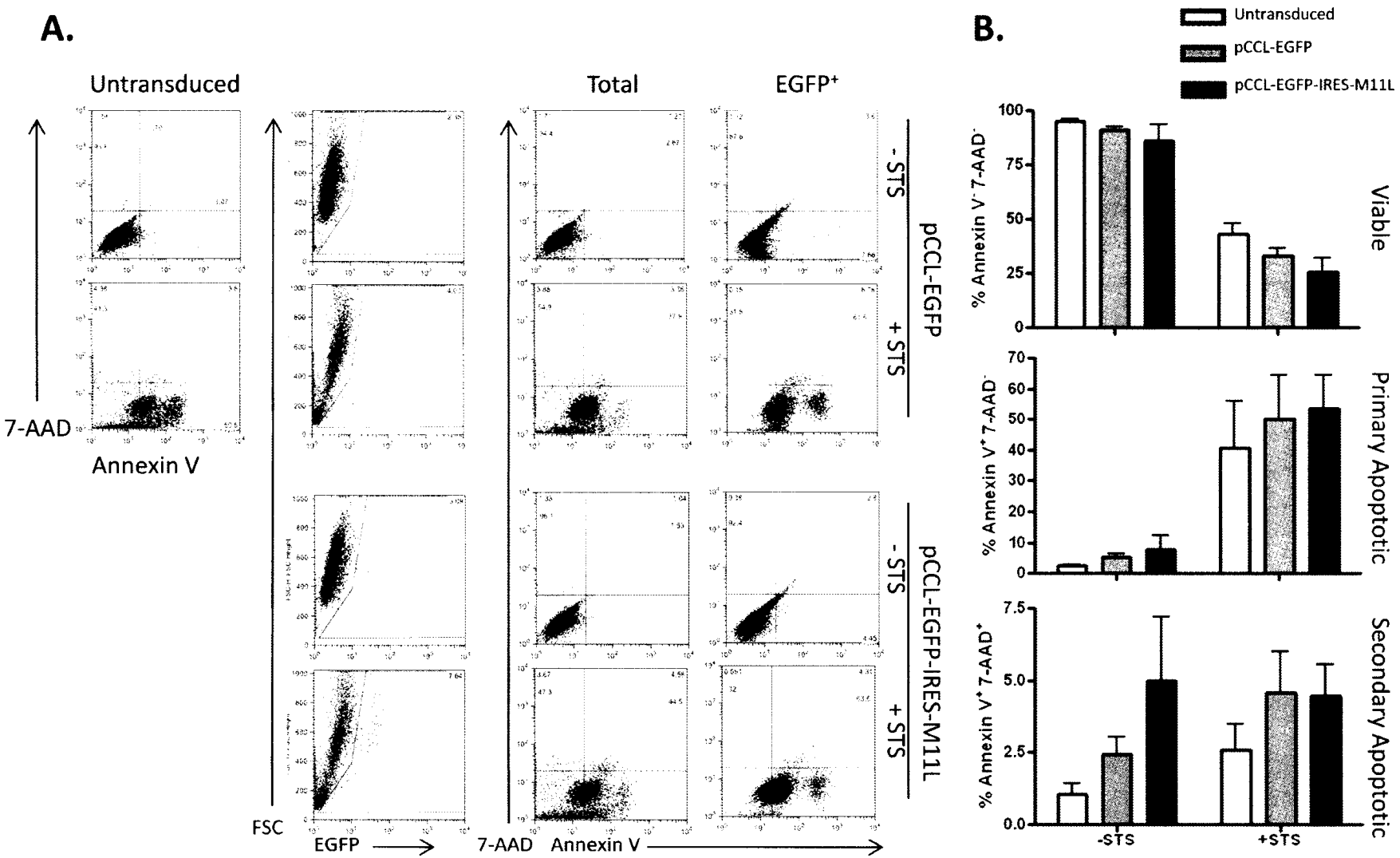

EGFP MFI
200
100
0
EGFP MFI
200
100
0
Untransduced
pCCL-EGFP
pCCL-EGFP-IRES-M11L
pCCL-EGFP-minCMV-hPGK-Luc
pCCL-EGFP-minCMV-hPGK-M11L


B.
Untransduced
pHERO-M11L-gp140-CpG-EGFP
pCCL-EGFP
pCCL-EGFP-RES-M11L
pCCL-EGFP-minCMV-hPGK-Luc
pCCL-EGFP-minCMV-hPGK-M11L
M11L
GAPDH
Lane 1 2 3 4 5 6

apoptotic properties of M11L (143), , I transduced rat L cells with pCCL-EGFP or pCCL-EGFP-IRES-M11L at an MOI of 5. Transduced and non-transduced cells were treated with 4μM STS or a DMSO control for 8 hours, 48 hours following transduction. STS functions as a general kinase inhibitor, leading to apoptosis through the intrinsic pathway. In order to specifically examine the population of transduced cells, I gated on $EGFP^+$ cells (as shown on Figure 8A Column 2). Levels of viability and apoptosis were determined by staining using annexin V and 7-AAD. Exclusion of both dyes represents viable cells, annexin V^+ cells are early apoptotic, and annexin V^+ $7\text{-}AAD^+$ cells are late apoptotic or already dead.

I first observed viability and apoptosis profiles in transduced and untransduced cell populations 48 hours after infection, in the absence of an apoptotic inducer (Fig 8). Untransduced cells were 95.3±0.8% viable (annexin V^- $7\text{-}AAD^-$), whereas pCCL-EGFP and pCCL-EGFP-IRES-M11L transductants were 90.8±1.7% and 86.2±7.4% viable respectively. The high initial viability was also reflected in a small population of early and late apoptotic cells. Untransduced cultures were 2.4±0.4% annexin V^+ $7\text{-}AAD^-$, in comparison to the pCCL-EGFP transductants, 5.3±1.2%, and pCCL-EGFP-IRES-M11L transductants which were 7.7±4.4%. Examination of the late apoptotic population showed sizes of 1.1±0.4% in untransduced cells, 2.4±0.6% in pCCL-EGFP transductants, and 5.0±2.2% in pCCL-EGFP-IRES-M11L transductants. None of the differences were observed to be significant following two way ANOVA testing. These results show that the transduction process did not result in any significant insult to viability of the cells.

Figure 8 – Viability profiles of transduced L cells following staurosporine induced apoptosis. Mouse L cells were transduced with pCCL-EGFP or pCCL-EGFP-IRES-M11L at an MOI of 5. Two days following transduction, L cells were cultured in complete DMEM containing 4μM STS for 8 hours. Following this, cells were washed and stained using 7-AAD and annexin V to detect apoptosis. pCCL-EGFP and pCCL-EGFP-IRES-M11L cells were previously gated on the $EGFP^+$ population. (A) Representative data showing viability profiles of treated cells. Column 1 shows viability profile of untransduced cells. Column 2 shows gating of $EGFP^+$ populations in transduced cell populations. Column 3 shows viability profile of total cell population in transduced cultures. Column 4 shows viability profile of $EGFP^+$ cells only in transduced populations based on gating in column 2. (B) Graph of pooled data. Representative of n=3 independent experiments. Two way ANOVA testing was performed to determine any significant differences and interactions between drug treated and transduced groups.


A.
Untransduced
Total
EGFP+
7-AAD
Annexin V
FSC
EGFP
7-AAD
Annexin V
- STS
+ STS
pCCL-EGFP
pCCL-EGFP-IRES-M11L
B.
Untransduced
pCCL-EGFP
pCCL-EGFP-IRES-M11L
% Annexin V- 7-AAD-
% Annexin V+ 7-AAD-
% Annexin V+ 7-AAD+
Viable
Primary Apoptotic
Secondary Apoptotic
-STS
+STS

Next I examined the effects of STS treatment on the untransduced and transduced cultures (Fig. 8A rows 2 and 4). Treatment with STS resulted in a large decrease in viability in all cells. Untransduced cultures were 43.0±5.1% viable, 40.7±15.5% early apoptotic, and 2.6±0.9% late apoptotic. The pCCL-EGFP transductants decreased to 33.0±3.6% viable, an increased to 50.3±14.3% early apoptotic and 4.6±1.4% late apoptotic. Similarly, the pCCL-EGFP-IRES-M11L transductants were 25.4±6.6% viable, 53.4±11.0% early apoptotic and 4.5±1.1% late apoptotic. However, I did not observe significant differences in viability or apoptosis levels between untransduced and transduced populations.

Short Term Effects of M11L Transduction

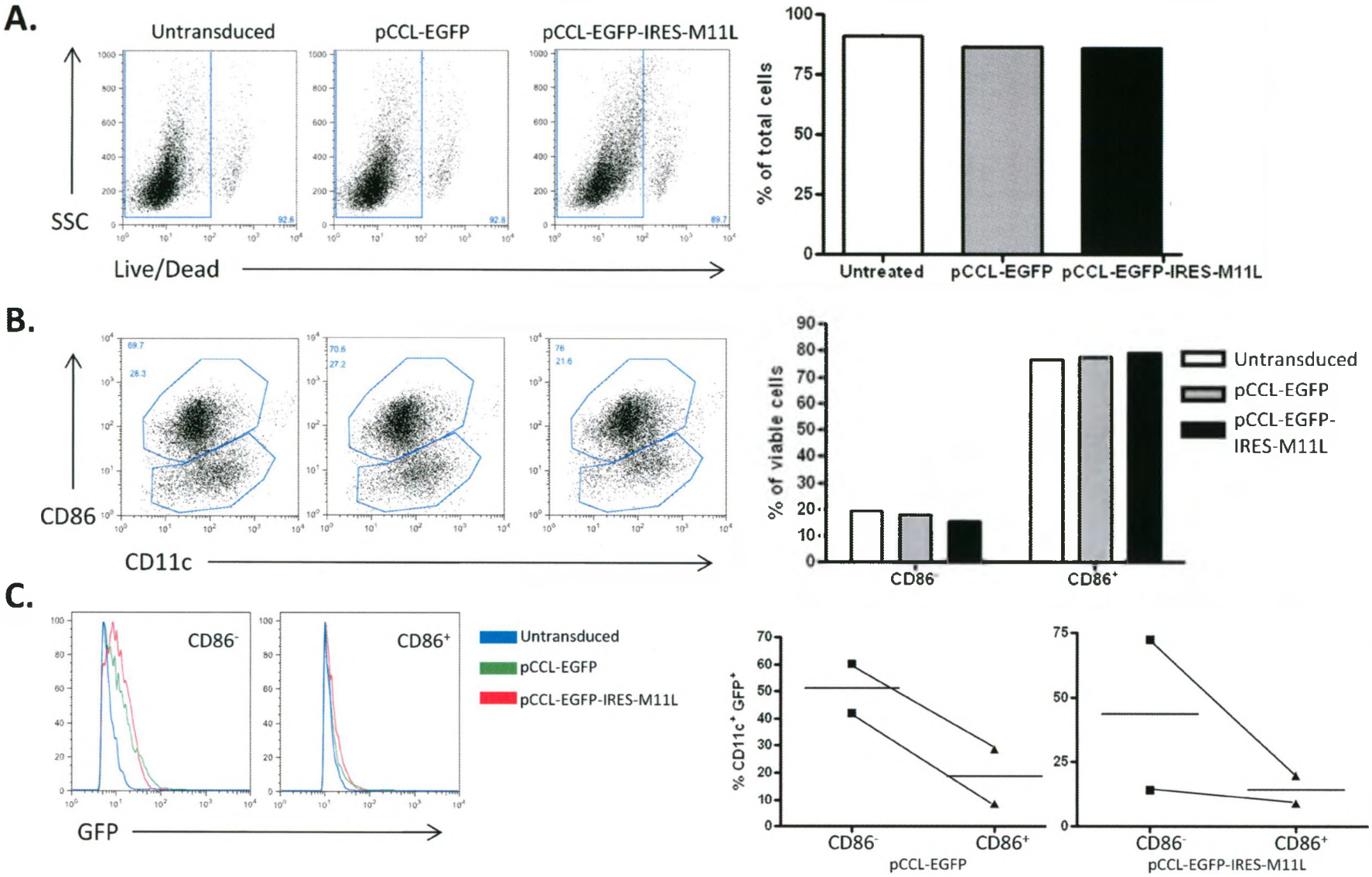
As mature DCs are required for immunogenicity, I wanted to determine if the transduction process affected the relative proportions of mature and immature DC populations, and if it decreased viability of these populations in the short term. This determination would allow us to observe if the transduction process resulted in an alteration of the cytokine environment that in turn would influence viability or the maturation profile independently of the genes being transduced. To do so, bone marrow cells were isolated from femurs and tibias of mice, cultured in IL-4 and GM-CSF and from these cultures, BMDCs were enriched 4 days later. Immediately following enrichment, DCs were transduced with the lentiviral vector. To begin, my studies focused on the pCCL-EGFP or pCCL-EGFP-M11L vectors. Infection proceeded for 24 hours, following which

effects of virus treatment on the BMDC populations were assessed by examining viability and maturation via CD11c and CD86 expression.

As viability was a major problem using non-viral vector-mediated transfection, I first assessed whether transduction also had the same negative effects (Fig. 9, top row). To measure this, the BMDC cultures were stained with a fluorescent vital dye taken up by dead cells and whole culture viability was examined by flow cytometry. Untransduced cultures were 92.7 % viable. Transduction did not appear to have an effect on viability, as viability levels of 92.4% and 90.6% were observed when using the pCCL-EGFP and pCCL-EGFP-IRES-M11L vectors respectively.

Since the maturation state of the BMDCs is relevant to their capacity to activate T cells, I examined the relative proportions of immature $CD11c^{+}CD86^{-}$ and mature $CD11c^{+}CD86^{+}$ in total DC cultures described above (Fig. 9B). Untransduced DCs were seen to be 76.6% mature and 19.3% immature. Transduction with either vector did not appear to have a large impact on maturation, as pCCL-EGFP transduced cultures had a mature to immature ratio of 77.7% to 18.0%. Additionally, M11L expression did not appear to increase maturation as pCCL-EGFP-IRES-M11L transduced cultures were seen to be 79.3% mature and 15.5% mature. These results suggest that transduction with these vectors had little to no effect on viability or the proportion of mature and immature cells in the total DC population.

Figure 9 – Lentiviral transduction process does not affect DC maturation or viability. Primary murine BMDCs were isolated and transduced with pCCL-EGFP or pCCL-EGFP-IRES-M11L. Cells were harvested 24 hours following transduction and analyzed by flow cytometry for EGFP expression, viability and cell markers CD86 and CD11c. (A) Representative data showing analysis of viability of the DC cultures. At right, pooled data representative of n=2 independent experiments. One way ANOVA testing was performed to determine any significant differences. (B) Representative data showing the gating of mature and immature DC populations based on CD11c and CD86 expression. Graph at right shows pooled data. One way ANOVA testing was performed independently on $CD86^-$ and $CD86^+$ populations to determine any significant differences from the use of any vectors. (C) Representative data showing histograms of GFP expression in mature and immature DC fractions of pCCL-EGFP and pCCL-EGFP-IRES-M11L treated cultures. Scatter plot at right shows data from 2 experiments. Lines connect matched pairs. Horizontal bar represents means of data.


A.
Untransduced
pCCL-EGFP
pCCL-EGFP-IRES-M11L
SSC
Live/Dead
% of total cells
Untreated
pCCL-EGFP
pCCL-EGFP-IRES-M11L
B.
CD86
CD11c
% of viable cells
CD86⁻
CD86⁺
Untransduced
pCCL-EGFP
pCCL-EGFP-IRES-M11L
C.
CD86⁻
CD86⁺
GFP
Untransduced
pCCL-EGFP
pCCL-EGFP-IRES-M11L
% CD11c⁺ GFP⁺
CD86⁻
CD86⁺
pCCL-EGFP
pCCL-EGFP-IRES-M11L

I also examined the level of EGFP expression in the mature and immature fractions (Fig. 9C). I observed that the pCCL-EGFP lentiviral vector transduced 51% of $CD11c^{+}CD86^{-}$ cells and 18.7% of $CD11c^{+}CD86^{+}$ cells. The pCCL-EGFP-IRES-M11L vector also displayed an increased level of transduction in immature cells, 43.5%, compared to mature cells, 14.5%. Although the differences were not seen to be statistically different, there is a consistent trend towards transduction of predominantly immature cells.

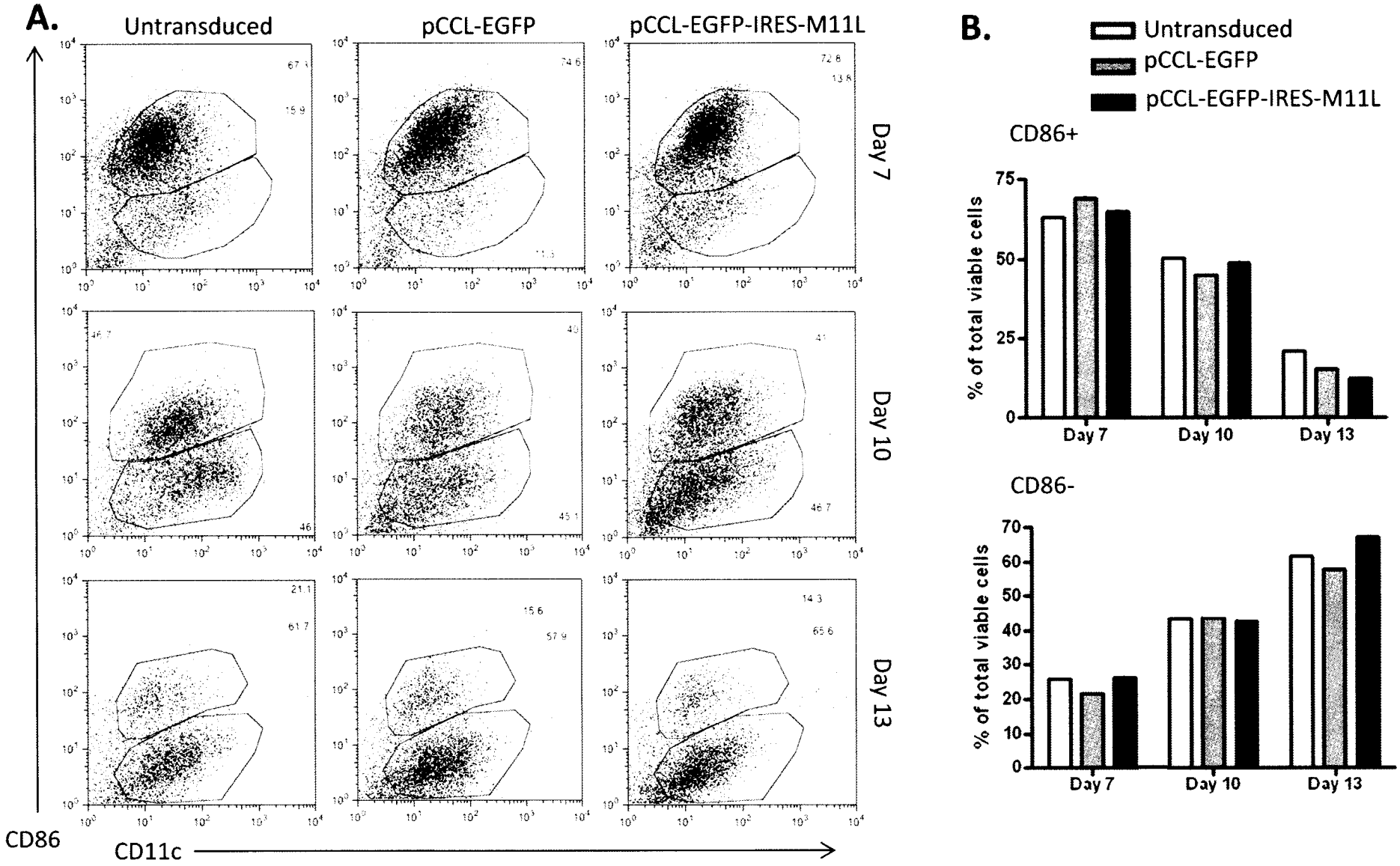
Long Term Culture of Bone Marrow-derived DCs

Owing to the potential ability of M11L to protect BMDCs from apoptosis, I performed long term cultures of primary BMDCs to observe any effects of M11L transduction on longevity *in vitro*. To do so, bone marrow was harvested from femurs and tibias of mice, and differentiation into DCs was induced with IL-4 and GM-CSF and enriched for DCs 4 days later. Immediately following enrichment, cells were transduced with pCCL-EGFP and pCCL-EGFP-IRES-M11L. The transduced cells were then maintained in culture up to day 13, with media and cytokines (GM-CSF and IL-4) replenished every 3 days after enrichment. At days 7, 10, and 13, cells were harvested and analyzed based on viability, and maturation state via flow cytometry. I analyzed the effects of transduction from two perspectives. Firstly, I determined if there were long-term effects of the transduction process on the total culture. Secondly, I specifically examined $EGFP^{+}$ cells to determine the effects of M11L transduction on longevity of the BMDCs.

To determine if the virus transduction process affected maturation of the cultures in long-term, I examined the proportions of $CD86^-$ and $CD86^+$ populations in untransduced and transduced cultures (Fig. 10). To do this, I gated on viable cells $CD11c^+$ and examined the distribution of $CD11c^+$ $CD86^+$ and $CD86^-$ cells. In the untransduced cultures, the immature $CD86^-$ population initially comprised approximately 25.9% of total viable cells while the mature population comprised 63.2% of viable cells. The mature population steadily decreased over the course of the culture, eventually falling to approximately 21.1%. Conversely, the immature population comprised more of the total viable cells by day 13, making approximately 61.7% of viable cells. Transduction with either virus did not appear to influence the maturation profile. Cultures transduced with pCCL-EGFP were initially 69.0% mature and 21.8% immature. At day 13, the profile switched to 15.6% mature and 57.9% immature. In the case of pCCL-EGFP-IRES-M11L transduced cultures, the initial profile was 64.9% mature and 26.1% immature. As with the other cultures, there was a shift towards the immature fraction, becoming 12.6% mature and 67.3% immature. These results may suggest increased cell death among the mature population. As I observed in the short-term study, there does not appear to be any lingering effects of the transduction process in the long-term study.

Due to the importance of maturation state in a DC vaccine, I examined the levels of EGFP expressing $CD11c^+$ $CD86^+$ and $CD11c^+$ $CD86^-$ cells based on the gates shown in figure 10 over the time course to see the levels of transduction in mature and immature fractions in the total culture (Fig. 11). The percentage of

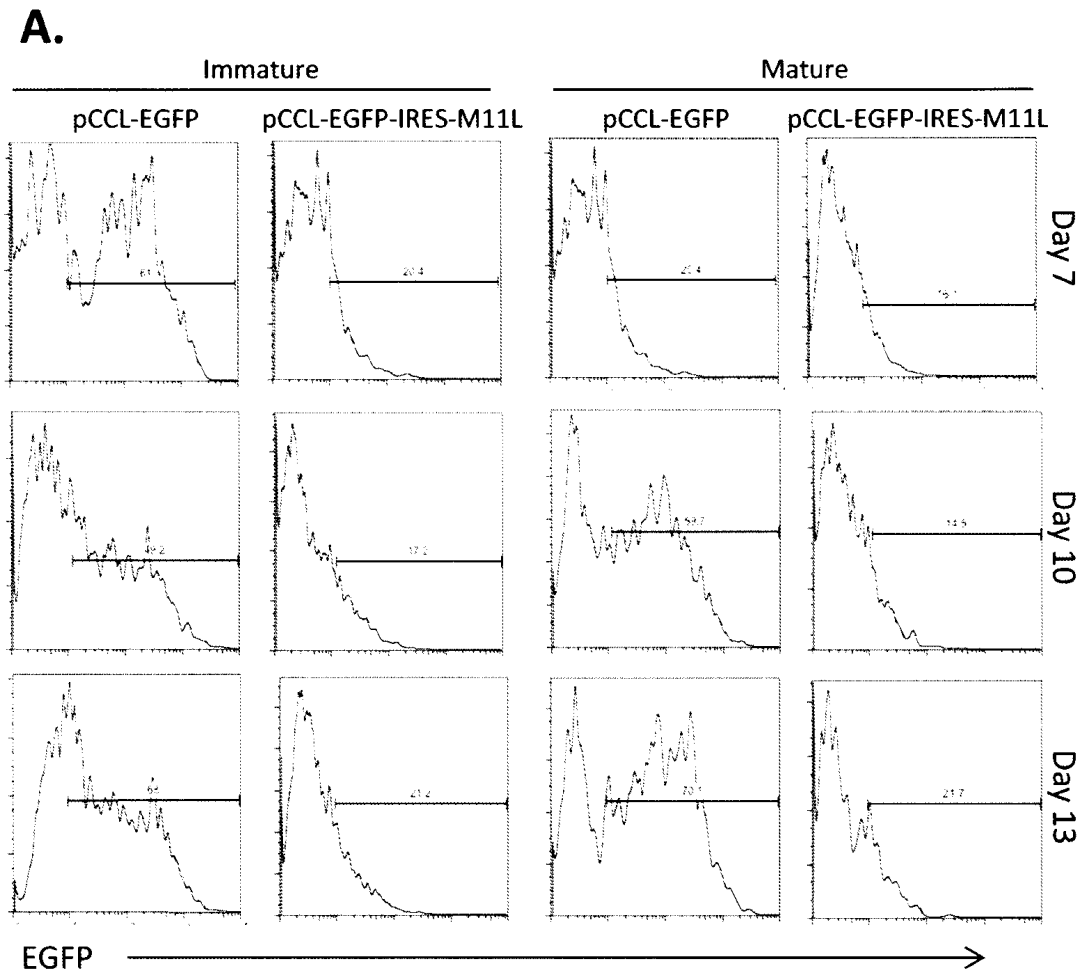
Figure 10 – The process of virus transduction does not have a detectable affect on DC maturation in the long term. Primary murine BMDCs were isolated and transduced 4 days later with pCCL-EGFP or pCCL-EGFP-IRES-M11L. Cells were examined at days 7, 10, and 13 post transduction by flow cytometry for CD11c and CD86 expression. (A) Representative data showing CD86 and CD11c staining in DC cultures collected at the time point shown (days post BMDC isolation). (B) Graph representing pooled data. Day 7 and 10 are representative of 2 experiments. Day 13 representative of 1 experiment. One way ANOVA testing was performed between the three groups on each day to determine any significant differences.

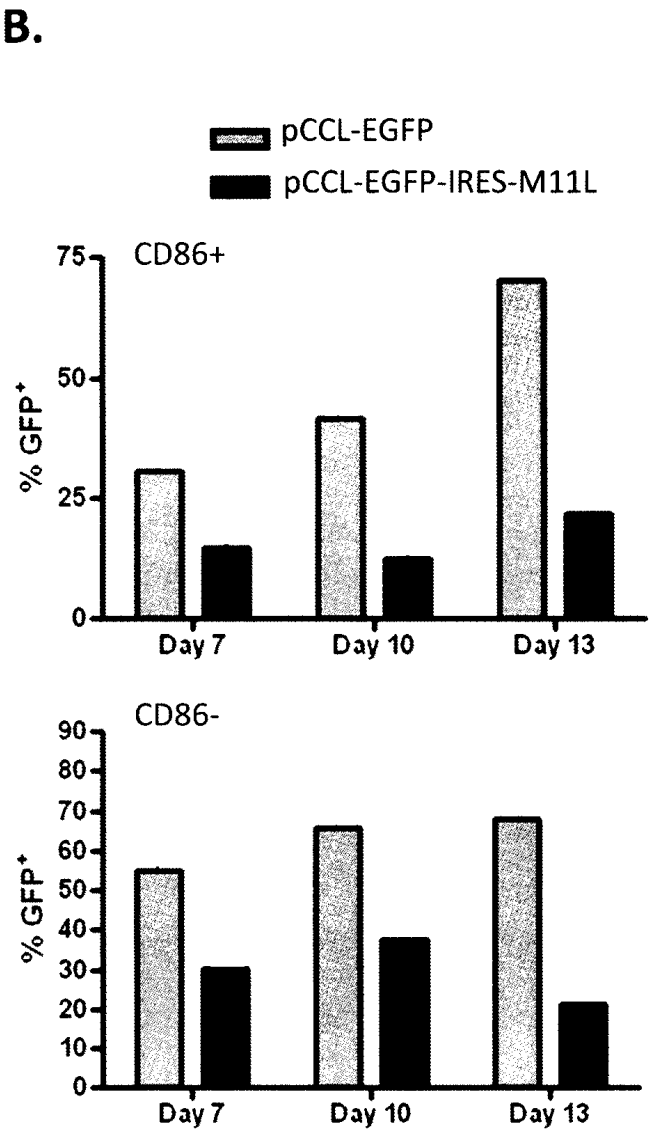

A.
Untransduced
pCCL-EGFP
pCCL-EGFP-IRES-M11L
Day 7
Day 10
Day 13
CD86
CD11c
B.
Untransduced
pCCL-EGFP
pCCL-EGFP-IRES-M11L
CD86+
% of total viable cells
75
50
25
0
Day 7
Day 10
Day 13
CD86-
% of total viable cells
70
60
50
40
30
20
10
0
Day 7
Day 10
Day 13

immature $EGFP^+$ pCCL-EGFP transductants increased from 55.0% to 68% by the end of the culture. However, there was a greater increase in the percentage of $EGFP^+$ mature cells, which increased to 70.1% from 30.9%. In the case of pCCL-EGFP-IRES-M11L transduced cultures, the level of $EGFP^+$ immature cells showed an overall decrease to 21.2% from an initial level of 30.2%. Conversely, the level of $EGFP^+$ mature cells increased from 14.7% to 21.7%. Repeats were performed using different virus stocks, possibly resulting in the variable levels of transduction. However, consistent to all trials was the difference in EGFP MFI observed when comparing pCCL-EGFP transduced cells to pCCL-EGFP-IRES-M11L transductants.

To determine if transduction of DCs with M11L resulted in less cell death, I examined the change in viability of both transduced and untransduced populations over the 13 day time course in the same population of cells analyzed in Figures 10 and 11 (Fig. 12A, top graph). I first examined the rate of death in the total culture to see if globally, all cultures were dying at approximately the same rate. To do so, I gated on total $CD11c^+$ cells. The untransduced culture decreased in viability from 80.3% to 46.5% at day 13. The transduced cultures also had a similar day 7 viability, 79.6% for pCCL-EGFP and 76.3% for pCCL-EGFP-IRES-M11L). As with the untransduced cultures, there was a drop in viability by day 13, as pCCL-EGFP transduced cultures were 43.4% viable, and pCCL-EGFP-IRES-M11L transduced cultures were 47.3% viable. This suggests that from a general outlook, each culture was dying at the same rate. Following

Figure 11 – The level of GFP^+ mature DCs increases over time. Primary murine BMDCs were isolated and transduced 4 days later with pCCL-EGFP or pCCL-EGFP-IRES-M11L. Cells were examined at days 7, 10, and 13 post transduction by flow cytometry for EGFP expression in the mature $CD86^+$ and immature $CD86^-$ populations. (A) Representative data showing the level of EGFP expression in pCCL-EGFP and pCCL-EGFP-IRES-M11L mature and immature cells. (B) Graph representing pooled data. Day 7 and 10 are representative of 2 experiments. Day 13 representative of 1 experiment.


A.
Immature
Mature
pCCL-EGFP
pCCL-EGFP-IRES-M11L
pCCL-EGFP
pCCL-EGFP-IRES-M11L
Day 7
Day 10
Day 13
EGFP


B.
pCCL-EGFP
pCCL-EGFP-IRES-M11L
CD86+
% GFP+
75
50
25
0
Day 7
Day 10
Day 13
CD86-
% GFP+
90
80
70
60
50
40
30
20
10
0
Day 7
Day 10
Day 13

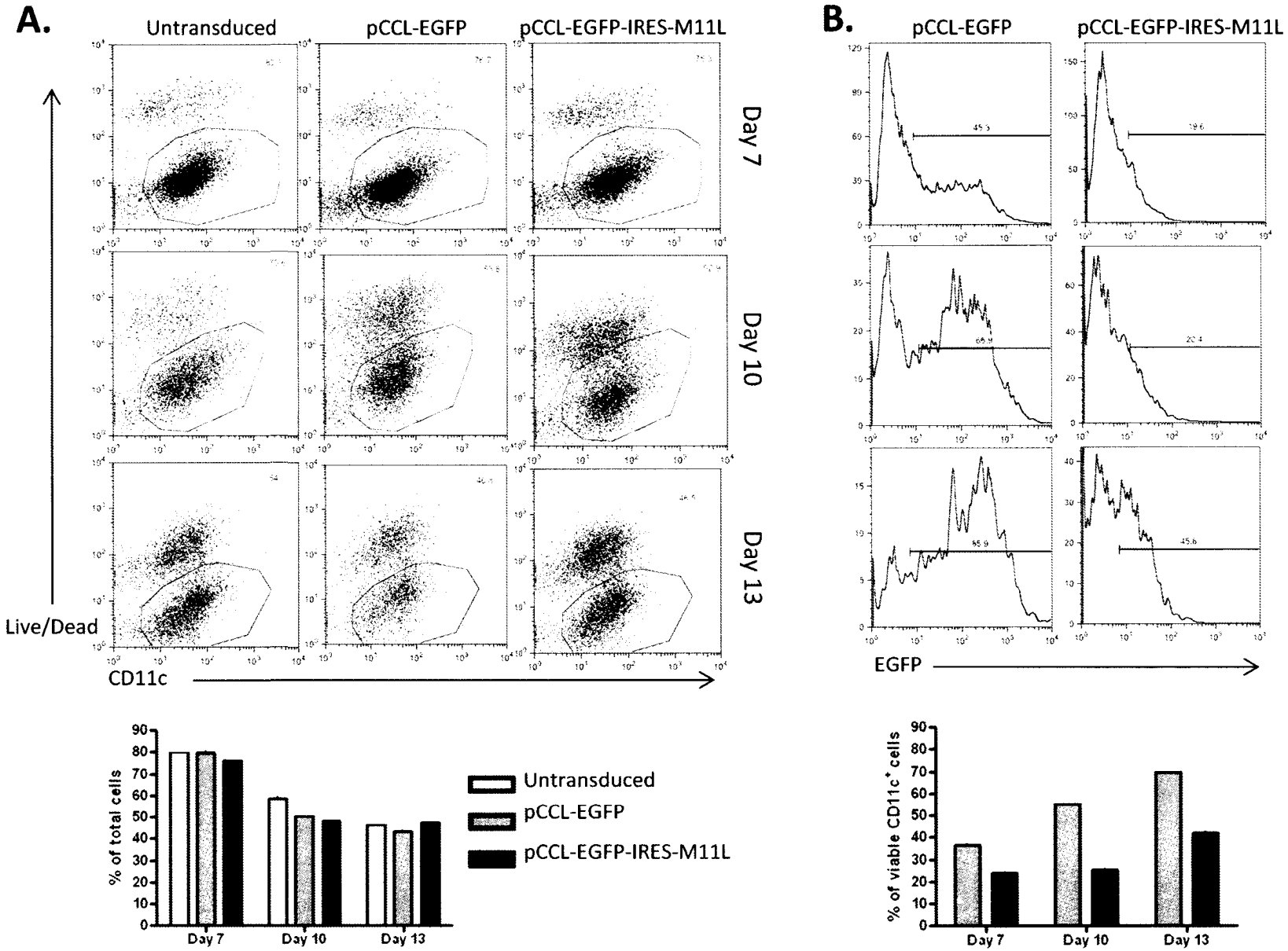
this observation, I wanted to see specifically how levels of transduced $EGFP^+$ cells related to the decrease in total culture viability.

I next examined the persistence of transduced $EGFP^+$ DCs in the culture (Fig. 12B). To do so, I gated on viable $CD11c^+$ cells and measured the level of transductants up to day 13. The pCCL-EGFP transductants comprised 36.7% of viable $CD11c^+$ cells at day 7, and increased to 69.7% at day 13. Conversely, pCCL-EGFP-IRES-M11L transductants were initially 28.0% of viable $CD11c^+$ cells, increasing to 42.0%. However, the sizes of both $EGFP^+$ populations appear to trend towards an increase as the average population size nearly doubles. It would appear as though in the long term study, the transduced cells persist longer; however M11L expression does not appear to have a significant effect.

DC Maturation Cocktail Does Not Appear to Increase Mature Transductants

The importance of mature DCs in modulating the immune response is well understood. However, I have observed in my transduction studies that the level of $EGFP^+$ $CD11c^+$ $CD86^+$ population was lower than that of the $EGFP^+$ $CD11c^+$ $CD86^-$. Therefore, I attempted to see if the application of a maturation cocktail to the DC cultures following transduction would increase the number of mature transductants. To do this, I treated the cells with a previously characterized maturation cocktail consisting of IL-1β, TNF-α, IL-6, CpG, and PGE2, 24 hours after transduction; thus, transduced immature cells would undergo maturation. The DCs were subjected to the maturation cocktail for another 16 hours prior to analysis by flow cytometry.

Figure 12 – Effect of M11L expression on DC viability *in vitro*. Primary murine BMDCs were isolated and transduced 4 days later with pCCL-EGFP or pCCL-EGFP-IRES-M11L. Cells were examined at days 7, 10, and 13 post transduction by flow cytometry for EGFP expression in the mature $CD86^{+}$ and immature $CD86^{-}$ populations. (A) Representative data showing gating of viable $CD11c^{+}$ cells at each day, and graph shows pooled data from n=2 independent experiments. (B) Representative data showing EGFP expression in viable $CD11c^{+}$ populations. Graph shows pooled data from n=2 independent experiments

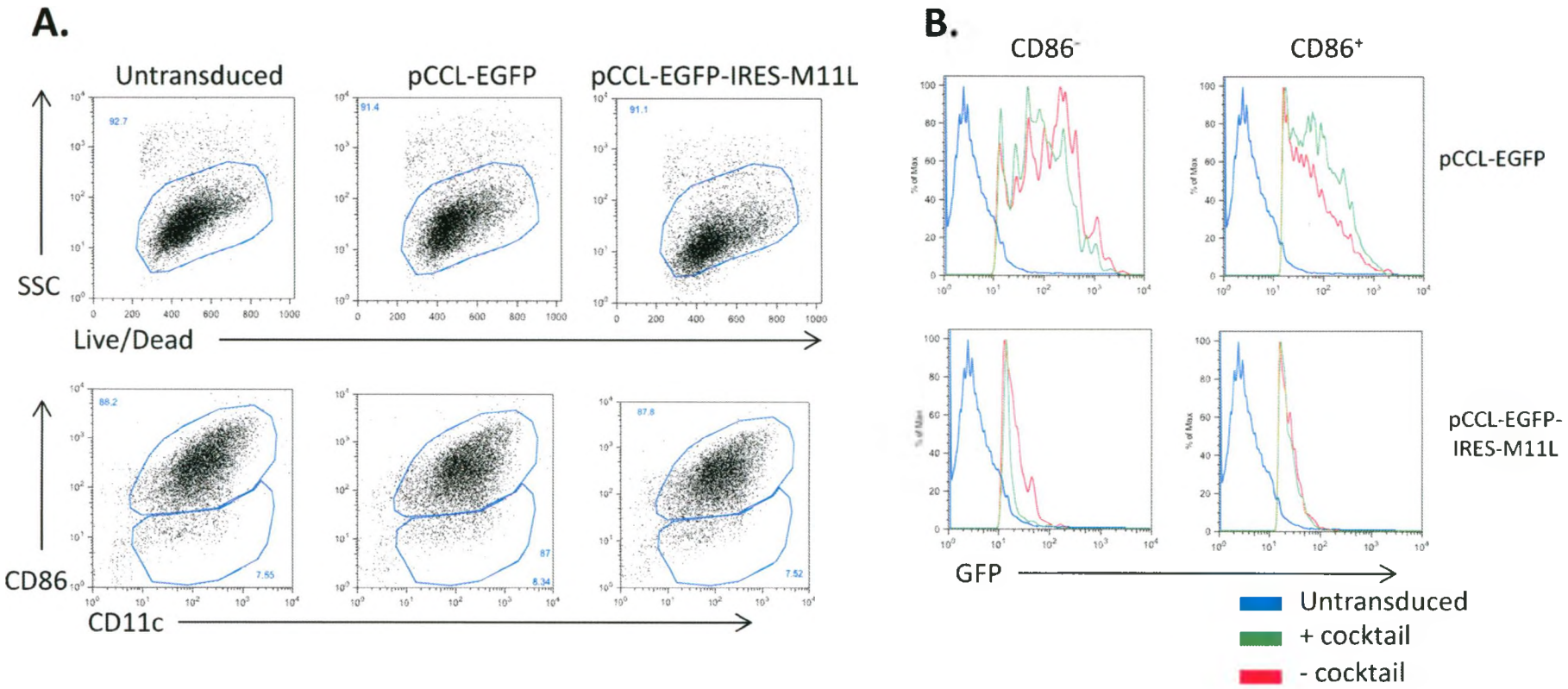

A.
Untransduced
pCCL-EGFP
pCCL-EGFP-IRES-M11L
Day 7
Day 10
Day 13
Live/Dead
CD11c
B.
pCCL-EGFP
pCCL-EGFP-IRES-M11L
EGFP
% of total cells
Day 7
Day 10
Day 13
Untransduced
pCCL-EGFP
pCCL-EGFP-IRES-M11L
% of viable CD11c+ cells
Day 7
Day 10
Day 13

I observed that administration of the maturation cocktail did not affect viability of the cells (Fig. 13A, top row). Untransduced DCs treated with the cocktail were observed to be 92.7% viable. Transduction with pCCL-EGFP resulted in 91.4% viable cells, and pCCL-EGFP-IRES-M11L treated cultures were 91.1% viable. As these numbers are similar to viability levels of DC cultures 24 hours post-transduction, it appears as though the cocktail did not affect viability. As expected, the maturation cocktail resulted in a shift towards a $CD86^{+}$ state (Fig. 13A, bottom row). Untransduced cultures shifted to 88.2% mature and 7.5% immature. The pCCL-EGFP transduced cultures were 87.0% mature and 8.3% immature. Lastly the pCCL-EGFP-IRES-M11L transductants were 87.8% mature and 7.5% immature.

Next, I examined the level of EGFP expression in the mature and immature fractions from a standpoint of transduction efficiency and level of EGFP expression by MFI (Fig. 13B). I observed that regardless of the lentiviral vector, higher numbers of transductants were seen in the immature fraction. In cultures without the maturation cocktail, the pCCL-EGFP vector transduced 60.1% immature cells and 28.9% mature cells. The pCCL-EGFP-M11L vector displayed a much lower transduction efficiency, at 14.4% mature cells, and 9.1%mature cells. In cocktail treated cultures, the pCCL-EGFP vector transduced 54.4% of immature cells and 32.7% of mature cells. The pCCL-EGFP-M11L vector overall again transduced fewer cells, 12.4% immature compared to 9.1% of mature cells.

The MFI resulting from pCCL-EGFP transduction was also higher in all cases. Taken together, these results demonstrate that the inclusion of the IRES-

Figure 13 – Administration of a maturation cocktail does not greatly increase the level of mature GFP^+ BMDCs. Primary murine BMDCs were isolated and transduced 4 days later with pCCL-EGFP or pCCL-EGFP-IRES-M11L. Twenty-four hours following transduction, BMDCs were cultured in the presence of a maturation cocktail consisting of IL-1β, TNF-α, IL-6, CpG, and PGE2 for 16 hours. Cells were then collected and analyzed by flow cytometry for viability, GFP, CD11c, and CD86. (A) Representative data showing gating of viable cells, and of mature and immature DC populations. (B) Histograms depicting GFP expression in pCCL-EGFP and pCCL-EGFP-IRES-M11L transduced cells in the presence and absence of cocktail. Data is representative of one experiment.


A.
Untransduced
pCCL-EGFP
pCCL-EGFP-IRES-M11L
SSC
Live/Dead
CD86
CD11c
92.7
91.4
91.1
88.2
7.55
87
6.34
87.8
7.52
B.
CD86⁻
CD86⁺
pCCL-EGFP
pCCL-EGFP-
IRES-M11L
GFP
Untransduced
+ cocktail
- cocktail

M11L sequence negatively affects the level of transduction by the lentiviral vectors. Furthermore, regardless of the expression vector used, there appears to be a bias towards the infection of immature DCs. Use of the maturation cocktail does not appear to greatly influence the proportion on mature transductants.

Discussion

The high capacity of DCs to initiate an immune response makes them an attractive option for immunotherapy. However, an efficient gene transfer method is required in order to efficiently introduce the antigen into DCs for vaccination purposes. I have assessed both non-viral and viral vector-mediated methods of gene transfer and observed that viral-vector mediated gene transfer provided the best balance between viability and efficiency of what????.. Using the pCCL-EGFP vector, I observed upwards of 40% transduction efficiency 48 hours after infection in BMDCs. However, the IRES-containing bicistronic vector appeared to transduce DCs far less efficiently, transducing slightly more than 10% of DCs at 48 hours post-infection. Curiously, I observed that pCCL-EGFP-IRES-M11L expressed EGFP at considerably reduced levels as compared with pCCL-EGFP. From my results, it is unclear if this is a direct result of M11L expression, the presence of an IRES, or the size of the integration cassette. Transduction with any of the viral vectors did not result in a large decrease in viability. Conversely, I have shown that conventional transfection reagents such as Lipofectamine or Turbofect do not mediate a high transfection efficiency of DCs. In my trials, I never observed transfection efficiencies greater than 5% with either method, while viability was a major problem also. Nucleofection resulted in the highest transfection efficiency of the three methods tested. However, it also had the lowest viability. This result coupled with the limited number of cells one can use in a single transfection, greatly limits the overall number of transfected cells that

expression by Langerhans cells results in the maturation of these cells, their migration to the lymph nodes, and induction of an HIV-specific Th1 immune response. The system was initially characterized using a simian HIV-based DNA construct, which showed a decrease in viral rebound when administered with HAART therapy (181). Later studies showed that DermaVir was capable of inducing HIV-specific $CD8^+$ effector and central memory responses (182). This form of therapy has shown clinical potential, as it has been cleared for Phase II clinical trials.

In order to assess if the level of M11L expression in the generated vector is sufficient for protection from apoptosis, I used pharmacological induction of apoptosis. The previous characterization of M11L function made use of immortalized cells treated with STS to induce apoptosis (141). I observed that transduction with my viral vectors did not result in a decrease in viability of the target L cells. Following STS treatment, M11L expression did not appear to result in any protective effect as viability levels appeared to decrease but not significantly. Population sizes of the primary apoptotic (annexin V^+ 7-AAD^-) and secondary apoptotic (annexin V^+ 7-AAD^+) were also examined. I did not notice any significant differences arising from STS treatment following transduction in either population. However, the late apoptotic population in pCCL-EGFP-IRES-M11L transductants did not appear to change following STS treatment, whereas the same population in untransduced and pCCL-EGFP transduced cells increased nearly 2-fold. This result may suggest a slight protective role by M11L.

In future experiments, the STS treatment experiment can be replicated in primary BMDCs. Although DCs *in vivo* are triggered to undergo apoptosis via the extrinsic pathway due to Fas-FasL interactions with $CD8^+$ T cells, induction of apoptosis may result in clearer observations than the long term culture. A step further than this would be to use the Fas-FasL interactions in DCs to consolidate the effects of M11L on the intrinsic and extrinsic apoptotic pathways. However, as the intrinsic and extrinsic pathways converge at the level of the mitochondria, it would be expected that both STS treatment and Fas-FasL interactions in DCs would have similar results. Alternatively, apoptosis levels may also be measured by examining the levels of mitochondrial proteins associated with apoptosis, including cytochrome c which is released into the cytoplasm due to the effects of Bak and Bax.

The discovery of an efficient and safe method of gene delivery to DCs is only one obstacle in the generation of DC-based vaccines. The immune response generated from such a vaccine ultimately depends on maturation and activation of the genetically engineered DCs. Firstly, I observed that transduction of a primary BMDC population did not have an effect on the proportion of mature versus immature cells. This is in agreement with findings from other groups that also performed phenotypic studies of lentivirus transduced DCs (183). In one study, they made use of the same three plasmid system, including a GFP expression transfer vector. They observed no increase in the mean fluorescence of total culture CD86. Secondly, transduction with M11L did not influence the maturation state either. Interestingly, I consistently observed a greater portion of

the EGFP$^+$ cells residing within the immature CD86$^-$ population. This may be explained by as of yet unknown restriction factors present within the mature CD86$^+$ population or a bias towards the infection of immature cells. Similar mechanisms are observed in CD4$^+$ T cells, where their activation state can influence susceptibility to infection by HIV-1 (184). Research conducted in 2007 by Dong *et al.* demonstrated that HIV-1 infection may be restricted in some subsets of mature DCs (184). Specifically, they found that LPS or TNF-α-matured DCs restrict HIV infection post-entry, at the level of reverse transcription and integration. Other groups have reported the selective infection of a subset of nonmaturing DCs in human blood, and the prevention of maturation by reducing antigen expression in infected cells. If a similar process exists in murine DCs, it may be responsible for preventing efficient transduction of mature DCs.

I performed a long-term DC culture in order to determine if transduction or the expression of M11L would have any effects on the DCs up to 13 days following isolation. In my observations, all transductants appeared to persist longer than non-transduced cells. In the total DC populations, I observed a general decline in total culture viability. However, at later time points, the sizes of the transduced cell populations increased relative to the size of the viable CD11c$^+$ population. However, these effects appear to be minimal in these experiments as statistical significance was not observed. It is possible, however, that further differences may arise at later time points.

To circumvent problems with direct infection of mature DCs, I administered a previously characterized maturation cocktail. The rationale behind

this was that immature DCs would first be transduced with the viral vectors. The experiment I performed consisted of an initial transduction, followed 24 hours later by administration of the maturation cocktail. Because of the HIV-restrictive nature of TNF-α matured DCs (184), it appears unlikely that a reversal of the order of transduction and cocktail administration would increase $EGFP^+$ $CD86^+$ cells. Following this, administration of the maturation cocktail would push the transduced immature cells into the mature fraction. As expected, there was a shift in the overall DC population towards a $CD86^+$ mature state seen in both transduced and untransduced populations. Interestingly, I observed an increase in $EGFP^+$ $CD86^+$ cells in the populations transduced with pCCL-EGFP. This was concordant with a slight decrease in the $EGFP^+$ $CD86^-$ population. Another study by Toscano *et al.* showed that LPS treatment following lentivirus transduction of DCs resulted in a greater than 4 fold increase in CD86 expression (183). This may in part be explained by the fact that they assessed CD86 within the whole culture rather than specifically those DCs that were transduced. This phenomenon was not observed in cells transduced with pCCL-EGFP-M11L; rather, the proportion of $CD86^-$ and $CD86^+$ cell populations did not change. Potentially, M11L may be exerting previously unknown effects on DC maturation. Such phenomena have yet to be examined as this is the first study that has attempted to express M11L in dendritic cells. However, it has been observed that pox viruses can inhibit DC maturation (185, 186). In order to determine the effectiveness of M11L inclusion, it will be necessary to determine if it has an

additional role in preventing DC maturation, as this has not been previously studied.

As the maturation state of the DCs will dictate its effectiveness in immunotherapy, it is important to determine a method that is suitable to mature or activate a large number of DCs. A recent method makes use of an inducible CD40 system (187). CD40, a TNF family receptor, normally interacts with its cognate ligand, CD40L expressed on $CD4^+$ T helper cells. The CD40-CD40L interaction results in increased antigen presentation and costimulatory capacity, as well as the synthesis of cytokines and anti-apoptotic molecules that all serve to enhance DC-mediated activation of $CD8^+$ T cells. In 2005, the group of Hanks *et al.* engineered an inducible CD40 system consisting of the cytoplasmic tail fused to a membrane bound drug binding domain (188). They demonstrated that primary BMDCs transduced with an adenovirus vector encoding the inducible receptor significantly increased levels of polarizing IL-12. Furthermore, use of inducible CD40 in a DC-based vaccine against OVA-peptide resulted in decreased EG.7-OVA tumor size. Co-expression of inducible CD40, M11L, and an immunogenic protein should provide an extremely robust immune response. However, one of the difficulties here would be the number of genes that have to be transferred. As observed in my studies, as well as others, gene transfer of one gene is already difficult. As such, it is still necessary to revise existing gene transfer methods to allow for triple gene synthesis.

Expression of M11L from the IRES may be resulting in levels that are too low to be effective (161). A possible cause for this may be the errors present in

the IRES sequence as this may affect IRES functionality. If the sequence variations occur in critical regions of the secondary structure formed by the IRES, loading of the translation initiation factors may be altered, potentially decreasing the level of translation. Based on my observations in HEK293T cells, however, complete IRES function did not appear to be abrogated as expression of M11L was verified in transduced cells. To verify that effective levels of M11L are being produced, M11L transduction can occur in parallel with myxoma virus infection to determine if M11L expression is comparable.

In order to examine this possibility, I have begun to study a second transfer vector system that makes use of two separate promoters oriented in opposite direction (164). Characterization of this system showed increased expression of the second gene. This may translate to an increased expression of M11L. Also, depending on the relative amounts of expression, it may also be possible to reverse the order of the genes encoded within the IRES containing transfer vector. In practice, this would depend on the level of expression required of an immunogenic peptide. In the case of GFP, studies have reported detectable levels of GFP expression when under the control of the EMCV IRES. A third possibility would be to use the foot and mouth disease virus (FMDV) protein 2A (189, 190). The 2A region acts as an intergenic cleavage site. Furthermore, the cleavage event does not require additional proteases, rather it is believed to occur due to conformational strain placed at the site of the 2A sequence during translation. However, when deciding on the method of dual gene expression, it is important to note that overexpression of M11L may also be cytotoxic. As such, it

may be necessary to explore other possible anti-apoptotic genes. Selecting the appropriate method for gene expression is not simply the one that results in the highest level of expression, but rather a fine balance must be obtained.

Two points raised by the transduction of these cells are the migratory capacity of the transduced DCs, as well as their actual persistence within regional lymph nodes. A phenotypic study of lentivirus transduced DCs showed that following transduction and LPS treatment, CCR7 expression increased, making the cells more responsive to lymph node-associated chemokine CCL19, suggesting normal migratory function (183). A separate group transduced DCs with an adenoviral vector expressing both EGFP and a hyperactive Bcl-x_L mutant and assessed both migration and persistence of the transduced DCs within the lymph node (127). They observed higher accumulation of DCs transduced with the Bcl-x_L mutant resulting in prolongation of the duration of DC presence within the lymph node. In agreement with previous studies, they also noted a decline in DC accumulation at the lymph node beginning 2 days after injection.

Once M11L expression is verified and levels of both genes within the transfer vector are optimized, further experiments can be performed to examine migration, persistence, and immunogenicity of the transgenic DCs. Previous research from my laboratory has shown that following footpad injection, PKH-green labeled DCs migrate to popliteal lymph nodes and persist for upwards of 4 days, peaking at day 2 post-injection. A similar schema can be performed using the transduced DCs. In order to separate transduced, non-transduced, and endogeneous DCs, the DC population to be injected can be labeled with PKH

red, prior to injection. I would expect to see results similar to those observed by the group of Yoshikawa *et al.* in 2008; similar migration levels of transduced and untransduced DCs were observed, but with increased persistence within the lymph node in the case of the former population (127). However, different methods of analysis can be performed to overcome the shortcomings of their quantitation techniques. While EGFP is necessary for the initial characterization and analysis of migration and persistence, it will be replaced with gp140 in order to assess immunogenicity of the DC vaccine in BALB/c mice, which exhibit known immune responses to the HIV envelope. Work here has begun as the appropriate pCCL-gp140 lentiviral vector has been generated.

To assess immunogenicity of the virus, it will be possible to use a variety of different proteins. However, due to the anti-apoptotic effects of M11L, the system should be able to accommodate what are normally cytotoxic proteins when overexpressed, notably the HIV *env* gene. The findings from the pHERO system demonstrate that M11L is able to increase immunogenicity of gp140 in the context of a DNA vector vaccine (143). In order to do this, the same schema can be performed as with the migration studies. However, instead of imaging lymph nodes, effector activity of splenic or lymph node T cells against the model epitope can be assessed *ex vivo*.

Summary

In my studies, I have observed the inefficiency of DC transfection using non-viral methods. As such, viral transduction was required in order to generate

transgenic DCs on a large enough scale. Consistent with previous findings, transduction of DCs does not appear to greatly affect viability and maturation. However, I have observed an increased propensity for the lentiviral vectors to transduce and exist in the immature DC population. M11L transduction did not appear to influence the relative proportions of mature and immature populations. However, its presence in the vector with the IRES did appear to decrease EGFP expression with respect to MFI and the number of transductants. M11L expression did not appear to have any major impact in viability in the long term culture, and this observation may have resulted from a level of expression too low to be effective. As a result, I have begun studying another bicistronic vector that should permit greater expression as it makes use of another promoter rather than an IRES. Optimization of the bicistronic vector and expression level of M11L will open doors for future studies making use of this DC-based system.

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Appendix

Appendix 1. Proof of ethics approval. A copy of the document showing official approval from the University Council on Animal Care for using the C57Bl/6 mice for DC studies.

THE UNIVERSITY OF WESTERN ONTARIO – ANIMAL USE SUBCOMMITTEE

PROTOCOL RENEWAL FORM

Protocol # 2007 049
Dekaban

A. PROJECT/INVESTIGATOR INFORMATION

Investigator Name: Gregory A. Dekaban	Current Protocol #: 2007 049
Project Title: *This is a NEW title* Yes ☐ In vivo MR imaging to assess delivery and efficacy of dendritic cell vaccines	

B. INVESTIGATOR DECLARATION

I. All animals used in this research project will be cared for in accordance with the recommendations of the Canadian Council on Animal Care and the requirements of the provincial legislation entitled, "The Animals for Research Act," of the Province of Ontario.

II. I confirm that the *Animal Use Protocol* accurately represents the proposed animal use.

III. I accept responsibility for procedures performed on animals on this project.

IV. I will ensure that any individual who will perform any animal-related procedure(s) within this protocol will complete all related mandatory training AND will be made familiar with the contents of this document.

1. I support the above declaration - YES ☒ Today's Date mm/dd/yy: May 27, 2010
2. By checking "YES" in this section, I authorize the submission of this form and its electronic delivery to awspc@uwo.ca
YES ☒ NO ☐
3. I authorize Christy Willert (PI Designate) to submit this form and to receive a copy of authorization via email on my behalf.
Today's Date mm/dd/yy: 05/27/10

Veterinary Use Only

Veterinary Authorization by: Click Here Authorization Date (mm/dd/yy): / /	Signature:

C. CHANGES AT RENEWAL CONFIRMATION – *Pick One Only*

☒ I confirm that NO CHANGES and/or NEW ELEMENTS to this AUP - other than staffing - have occurred since the last AUS Form submission.

☐ I confirm that all CHANGES and/or NEW ELEMENTS to this protocol not previously submitted WILL BE noted on a *Protocol Modification* form along with the *Protocol Renewal* Form.

D. THREE R's PROGRESS REPORT

Describe any progress made with respect to the Three R's of replacement, reduction and refinement of animal use during the PAST PROTOCOL YEAR. See the CCAC 3 R's Website: http://www.ccac.ca/en/3Rs/index.html

Although we typically only need blood samples and tissue organs from our mice as well, when training some one, make use of the blood and spleens from the mice in order to not have to order additional mice for training purposes.

☐ *No animals were used during the past Protocol Year.*
☒ *No additional animals were requested during the past Protocol Year.*

E. ANIMAL USE COMPLICATIONS PROGRESS REPORT

Describe any complications beyond the expected experimental outcomes and end points encountered relative to animal use – e.g. unexpected outcomes and any unexpected animal pain, distress or mortality – AND, describe measures being undertaken to resolve these complications for the PAST PROTOCOL YEAR.

☒ *No complications were encountered relative to animal use during the past Protocol Year.*

F. ANALGESIA ADMINISTRATION UPDATE

Choose One of the Following Statements:

1. Analgesics were administered as outlined within the *Animal Use Protocol* ☐ **If checked, please list all analgesics currently used below:*

2. Analgesics are not indicated in this *Animal Use Protocol* ☒

G. PROTOCOL PERSONNEL UPDATE

Complete for ALL Staff Working Under This Protocol for This Renewal Year

[Address] Ref: [illegible]

CCAC Mandated Training Requirements – All personnel working with animals require CCAC mandated training including the 'Basic Animal Care & Use Web-CT Course' and related hands-on 'Workshops'. Completion of the 'Basic Animal Care & Use Web-CT Course' once every 3 years is mandatory for ALL personnel, including the Principal Investigator. The Animal Use Subcommittee will be informed of all personnel with incomplete training beyond 1 month of notice.

Workshop Enrolment Detail – The 'Workshop' requirements are determined by the species and procedures associated with each individual listed below. All personnel listed below will be contacted directly via the email address listed below for auto-enrolment in all 'Workshop' requirements. Previous hands-on 'Workshops' attended at another research institution may be accepted; please submit training documentation with this form. For additional training requirement detail and associated costs, go to:
http://www.uwo.ca/animal/policy/CCAC/Content/Training_and_Courses.htm

COMPLETE ALL COLUMNS BELOW PER PERSON					If "YES" to HANDS-ON ANIMAL WORK, Complete This Section PER SPECIES										
FIRST NAME *Repeat For Each Species Used*	LAST NAME *Repeat For Each Species Used*	ROLE *Role within this Protocol* *Researcher Staff Student*	EMAIL Address *"Mandatory Field"* (UWO & Affiliated Institution Emails Preferred)	HANDS ON Animal Work? YES or NO	SPECIES One Species Per Row *Use an Additional Row for Each Species*	Expected START DATE mm/dd/yy	PROCEDURES PER SPECIES 1=Basic Handling 2=Health Monitoring 3=Blood Collection 4=Injections 5=Anaesthesia 6=Surgery-Recovery 7=Surgery-Non-Recovery 8=Euthanasia/Post Mortem 9=Other *Provide Detail Below, e.g. subcutaneous*									
							1	2	3	4	5	6	7	8	9
Gregory	Dekaban	Researcher	dekaban@robarts.ca	Yes	Mouse	06/15/10	☒	☒	☒	☒	☒	☐	☐	☒	☐
Paula	Foster	Researcher	pfoster@imaging.robarts.ca	Yes	Mouse	06/15/10	☒	☒	☒	☒	☒	☐	☐	☒	☐
Christy	Willert	Staff	cwillert@robarts.ca	Yes	Mouse	06/15/10	☒	☒	☒	☒	☒	☐	☐	☒	☐
Sonali	deChickera	Staff	sdechick@uwo.ca	Yes	Mouse	06/15/10	☒	☒	☒	☒	☒	☐	☐	☒	☐
Mia	Merrell	Staff	mmerrell@robarts.ca	Yes	Mouse	06/15/10	☒	☒	☒	☒	☒	☐	☐	☒	☐
Bryan	Au	Student	cau23@uwo.ca	Yes	Mouse	06/15/10	☒	☒	☒	☒	☒	☐	☐	☒	☐

EMERGENCY *AFTER HOURS* CONTACT NAMES & NUMBERS NO LAB PHONE NUMBERS	PRIMARY EMERGENCY CONTACT LAST NAME & INITIAL: Dekaban, G.A.	PRIMARY EMERGENCY CONTACT NUMBER (HOME OR CELL): (h) 519-472-4677 or (cell) 519-282-0642
	SECONDARY EMERGENCY CONTACT LAST NAME & INITIAL: Willert, C.	SECONDARY EMERGENCY CONTACT NUMBER (HOME OR CELL): 519-871-0397
If applicable, ——— POST-OP CARE personnel detail	☒ Same as Emergency Contact, or Name:	Emergency #: 519-472-4677 Lab #: 24281 Email: dekaban@robarts.ca
If applicable, provide MRI/Cardiac personnel detail	☒ Same as Emergency Contact, or Name:	Emergency #: 519-871-0397 Lab #: 24239 Email: cwillert@robarts.ca