DENDRITIC CELLS TRANSFECTED WITH AUTOLOGOUS SIV RNA: POTENTIAL AIDS VACCINE

by

Nada Mohamad Melhem

BS, American University of Beirut, 1991

MS, American University of Beirut, 1994

Submitted to the Graduate Faculty of

The Graduate School of Public Health in partial fulfillment

of the requirements for the degree of

Doctor of Philosophy

University of Pittsburgh

2007

UNIVERSITY OF PITTSBURGH

GRADUATE SCHOOL OF PUBLIC HEALTH

This dissertation was presented

by

Nada M. Melhem

It was defended on

September 20, 2007

and approved by

Michael Murphey-Corb, Ph.D.
Professor
Departments of Molecular Genetics and Biochemistry, School of Medicine
Department of Infectious Diseases and Microbiology, Graduate School of Public Health
University of Pittsburgh

Andrea Gambotto, M.D.
Assistant Professor
Departments of Surgery and Molecular Genetics and Biochemistry
School of Medicine
University of Pittsburgh

Pawel Kalinski, M.D., Ph.D.
Assistant Professor
Departments of Surgery and Immunology, School of Medicine
Department of Infectious Diseases and Microbiology, Graduate School of Public Health
University of Pittsburgh

Charles R. Rinaldo Jr., Ph.D.
Professor
Department of Infectious Diseases and Microbiology, Graduate School of Public Health
Department of Pathology, School of Medicine
Clinical Microbiology Laboratory, University of Pittsburgh Medical Center
University of Pittsburgh

Dissertation Advisor: Simon M. Barratt-Boyes, BVSc, Ph.D.
Associate Professor
Department of Infectious Diseases and Microbiology, Graduate School of Public Health
Center for Vaccine Research
Department of Immunology, School of M edicine
University of Pittsburgh

Simon M. Barratt-Boyes, BVSC, Ph.D.

DENDRITIC CELLS TRANSFECTED WITH AUTOLOGOUS SIV RNA: POTENTIAL AIDS VACCINE

Nada M. Melhem, Ph.D.

University of Pittsburgh, 2007

ABSTRACT

The need for a therapeutic human immunodeficiency virus (HIV) vaccine is urgent for the control of the acquired immunodeficiency syndrome (AIDS) epidemic. The variability of the virus as well as its ability to undergo escape mutations in T cell epitopes are important obstacles facing the development of an AIDS vaccine. Consequently, a powerful strategy would be the induction of robust antigen specific T cell responses targeting patient-specific virus sequences expressed during the course of infection. An attractive vaccine approach to achieve this is the use of dendritic cells (DC) transfected with *in vitro* transcribed mRNA encoding autologous virus sequences. The rhesus macaque model provides an ideal preclinical setting to test the therapeutic potential of DC-based vaccination. We hypothesize that optimal antigen presentation and stimulation of potent T cell responses could be achieved by loading DC from SIV-infected macaques with mRNA encoding virus-derived sequences isolated during the course of infection. This represents a powerful strategy for the generation of a potential therapeutic AIDS vaccine. In support of our hypothesis, we generated the following evidence: (1) nucleofection is a superior method for efficient transfection of human and monkey monocyte-derived DC with DNA and mRNA to conventional electroporation and lipofection; (2) nucleofection of DC with mRNA led to better protein expression and DC maturation as compared to DNA transfection; (3) mRNA

nucleofection of DC resulted in rapid and sustained gene expression, a critical factor in DC-based immunotherapy for durable antigen presentation; (4) nucleofection of monkey monocyte-derived DC with wild-type non codon-optimized *gag* mRNA was efficiently expressed and induced strong antigen-specific T cell responses whereas DNA transfection led to non-specific T cell stimulation; (5) enhanced $CD4^+$ T cell responses were observed when Gag was redirected to the lysosomal pathway *via* the targeting signal of the lysosome-associated membrane protein (LAMP-1) following nucelofection of DC with mRNA; (6) rhesus DC transfected with lysosome-targeted *gag* mRNA encoding an escape mutation in an immunodominant CTL epitope stimulated $CD4^+$ and $CD8^+$ T cell responses of almost equivalent magnitude directed towards undefined epitopes outside of the mutated region; (7) *gag* or *env* mRNA transfected-DC from SIV-infected macaques stimulated significant antigen-specific T cell responses in an entirely autologous system; (8) DC cotransfected with *gag* mRNA as well as mRNA encoding CD70 or OX40L did not result in enhanced immunostimulatory functions. HIV/AIDS is a significant public health problem demanding action. This work demonstrates that mRNA-transfected DC expressing SIV antigen from infected monkeys stimulate broad and relevant T cell responses, thus supporting this approach for the generation of a therapeutic HIV vaccine to decrease the burden associated with the infection.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ACKNOWLEDGEMENTS

I would like to thank my research advisor, Dr. Simon M. Barratt-Boyes for his guidance and for giving me the opportunity to formulate and implement my ideas independently.

I would like to acknowledge my committee members, Dr. Michael Murphey-Corb, Dr. Andrea Gambotto, Dr. Pawel Kalinski and Dr. Charles Rinaldo Jr. for challenging me and providing me with valuable suggestions during my graduate career.

I thank my laboratory members past and present for their daily support.

I would like to thank the faculty members at the Infectious Diseases and Microbiology Department as well as the staff.

Last but not least, I send my gratitude to my parents for their endless dedication and support and I thank them for what I am. I also want to thank my brothers and sisters: Ali, Nadine, Dina, Imad and Nadim for their continuous support and unconditional love. Ali and Nadine have been my best friends all along and have been involved in the ups and downs of my graduate career; I thank them for their presence. I thank Dina and Imad for caring about all my accomplishments. I also send my thanks to Nadim, talking to him always made things easier. I also want to thank my nephew Ali Jr. for making me smile in the most stressful times.

1.0 INTRODUCTION

1.1 HIV-1 AND SIV

Human immunodeficiency virus (HIV), the causative agent of acquired immunodeficiency syndrome (AIDS), belongs to the family *Retroviridae* and genus lentivirus. HIV-1 isolates are classified into 3 groups; M, O and N (135, 242). M is responsible for the majority of HIV-1 global epidemic and is further subdivided into subtypes or clades (A-K, excluding E). Simian immunodeficiency virus (SIV) is also a lentivirus. SIV is a species restricted to African nonhuman primates and does not cause disease in their natural host (109). Evidence indicates that recombination of distinct SIV isolates led to the generation of HIV-like viruses. These viruses infect wild chimpanzees but do not cause disease. It is suggested that humans have been infected zoonotically to initiate the AIDS epidemic. HIV-1 and SIV, responsible of the progressive failure of the immune system AIDS, share approximately 40-50% genomic sequence homology (107) and that serological cross-reactivity is limited (127).

There are several similarities between SIV infection of macaques and HIV-1 infection of humans. Similar to HIV, most SIV isolates use the CCR5 coreceptor for viral entry (271, 288, 290). In addition, both HIV-1 and SIV replicate in activated as well as resting T cells (291). Infection by HIV or SIV is characterized by rapid depletion of memory T cells in gut associated lymphoid tissues (34, 165, 168). Following infection of humans and macaques with HIV-1 and

SIV respectively, acute infection resolves with the onset of antigen-specific immune responses (31, 120, 136, 137, 235). Moreover, HIV-1 and SIV utilize similar evasion mechanisms to escape the host immune responses (44, 91, 92, 218, 277, 289). The plasma viral load in both infections predicts the rate of disease progression (154, 170). The onset of disease in infected individuals and macaques takes place after $CD4^+$ T cell depletion; progress to AIDS is accompanied by a variety of opportunistic infections, hematological as well as neurological disorders (150). Due to existing similarities between HIV and SIV, the SIV macaque model is the best animal model for studying HIV pathogenesis and testing possible therapeutic strategies (57, 132).

1.2 THE GLOBAL AIDS EPIDEMIC

Since 1981, more than 25 million people have died of AIDS. Moreover, the number of people living with HIV has risen from around 8 million in 1990 to 40 million today and is still growing. Around 4 million adults and children became infected with HIV during 2006. Despite improvements in access to antiretroviral therapy, 3 million people died of AIDS last year (www.unaids.org). Thus, HIV continues to be a burden of mortality and morbidity.

1.3 ANTIRETROVIRAL THERAPY AND HIV INFECTION

Highly active antiretroviral therapy (HAART) has revolutionized patient care with HIV. HAART maintains suppression of HIV-1 replication to very low levels for an extended period of time in HIV-1 infected individuals, as well as reduces the morbidity and mortality of infected patients.

Thus, the use of HAART promoted the quality of life by controlling viral replication in infected individuals (110, 203). However, HAART is accompanied by many problems. Long-term metabolic toxicities, development of drug resistance resulting in escape mutants, and the cost of drugs as well adherence to strict regimens are important factors leading to reduction in the long-term use of antiretroviral therapy (ART) (208). Another important factor contributing to failure of HAART in long term HIV-1 control is the persistence of latent virus in $CD4^+$ T cells, providing a life-long supply of replication-competent forms of HIV-1 (49, 75, 105) and thus rendering any hope for virus eradication unrealistic with current ART (50, 210). Moreover, it has been demonstrated that when drug treatment is interrupted, plasma viral loads rebound to pretreatment levels (60, 202). These findings, along with the persistence of HIV-1 in other reservoirs such as the central nervous system (87), and the inability of HAART to clear infection, urgently require an alternative approach to control long-term infection.

1.4 HIV INFECTION: IMPACT ON THE IMMUNE SYSTEM

1.4.1 Overview: immune responses to HIV

A burst of viremia is detected during the primary stage of HIV infection by day 7 and peaks 3 weeks after exposure. During primary infection with HIV-1, $CD8^+$ T cells are detectable in circulation before neutralizing antibodies and a decline in plasma viremia is associated with expansion of these cells (31, 136). The development of antibody responses takes place rapidly with increased affinity to early virus strains (218). A set point, or point of equilibrium, is reached 2 to 6 months post infection. Early in infection, HIV-1 forms a pool of latently infected, resting

$CD4^+$ T cells (49). As a result, HIV-1 infection is established indefinitely. Even when ART is capable of reducing viremia to undetectable levels (<50 RNA copies per milliliter), these viral reservoirs persist (50). Despite the generation of cellular and humoral immune responses, progression to AIDS still takes place. Therefore, the ultimate goal of HIV/AIDS research is the development of a safe and effective vaccine.

1.4.1.1 Control of HIV/SIV infection

When designing a vaccine, both the humoral and cellular immune responses should be elicited. However, the fact that HIV-neutralizing antibodies are detected several weeks post infection and the absence of temporal relationship between early viral control and antibodies generation raised questions regarding the importance of these antibodies in HIV control (148, 149).

Understanding the mechanisms by which HIV-1 replication is controlled is important for the design of successful therapeutic vaccines. It is well known that cell-mediated immune responses play important role in host defenses against viral infections. In particular, $CD8^+$ T cells play a critical role in the host defense mechanisms in HIV-infected individuals where the increase in cytotoxic T lymphocytes (CTL) activity coincides with the clearance of viremia (31, 136, 199) and the observation of low viral loads and persistent non-progressive infections (220). In rare individuals known as elite controllers, the specific cellular immune responses are associated with suppression of HIV replication to undetectable levels (25).

As in HIV-1 infection, primary infection of rhesus macaques with SIV is characterized by a burst of virus replication followed by a decline in viremia due to emergence of CTL (120, 137, 235). Importantly, depletion of $CD8^+$ lymphocytes in primary and chronic SIV infection, results in uncontrolled viremia and inability to generate neutralizing antibodies. These findings reflect

the strong antiviral activities of $CD8^+$ T cells and highlight the importance of high frequency CTL responses in the development of an efficient HIV/SIV vaccine.

In addition to the critical role played by $CD8^+$ CTL in the control of HIV-1 replication, $CD4^+$ T cells are also involved in the control of viral infection. This is achieved by providing help for the stimulation and/or maintenance of antibody as well as $CD8^+$ T cell responses (8). In addition, $CD4^+$ T cells mediate antiviral effector functions through cytokine production or cytolysis (194). In deed, a strong negative association has been found between viral load and Gag-specific $CD4^+$ T helper-cell responses (223). Moreover, Gag-specific T helper cells positively correlated with Gag-specific CTL and negatively correlated with plasma viral load. It has been demonstrated that virus-specific $CD8^+$ T cells persist in the absence of $CD4^+$ T cells but have low effector function (245). Consequently, the induction of HIV-1 -specific CTL as well as T-helper responses is an important criterion in the design and development of an effective AIDS vaccine (90, 124, 125).

1.5 HIV/AIDS VACCINE: CHALLENGES

The findings stated above emphasize the strong requirement of $CD4^+$ and $CD8^+$ T cells for the control of HIV-1 infection, and therapeutic vaccines designed to promote broad cellular immune responses are being actively pursued. However, an important challenge in the development of vaccines for HIV-1 is the diversity of clinical isolates (83) coupled with the propensity for the virus to undergo escape mutations in T cell epitopes through selective pressure *in vivo* (91, 92).

1.5.1 Genetic variability of HIV-1

Considerable heterogeneity is revealed by the phylogenetic tree analysis of HIV RNA and protein sequences (216). Genetic variability of HIV is due the rapid replication of the virus, its high mutation rate as well as propensity for recombination (133, 135). Due to emergence of virus variability during chronic HIV-1 infection, the immune system has to deal with a large number of viral variants rather than the original one seen in acute infection (274). There are three types of HIV-1, M, N, and O. M, the main type, is divided into 10 subtypes or clades. These clades differ by approximately 20% of amino acid sequence as well as their geographic distributions. Moreover, there is up to 10% variability in the amino acid sequences within the clades. Subtype C is the most prevalent HIV-1 globally, especially in regions where vaccines might be evaluated (UNAIDS, www.unaids.org). Although the hope is to generate a single vaccine strain inducing a cross-reactive response conferring protection, the relevant issue of viral diversity confinds this goal (149). Consequently, two major approaches have been used for the selection of a vaccine strain: the first is to use isolates of specific subtype from a geographic region where the vaccine is to be used (238), for example using Indian strain in India and so on. The second strategy is to construct a consensus or ancestral sequence on the basis of an evolutionary mode (83). Consensus sequences are central sequences to the virus based on the most common amino acid in each position in an alignment (135, 196). Based on maximum likelihood, ancestral sequences are reconstructed from a phylogenetic tree. These sequences are similar to the circulating strain and may elicit cross-reactive responses.

Depending on the protein under consideration, 5-25% of amino acids within viral strains differ within the same HIV-1 clade (274). This variability affects the cellular immune responses in infected individuals due to the constraints imposed by the sequence specificity (89, 206, 207).

It has been recently reported that the use of autologous peptides allows the detection of strong and broad T cell responses against HIV-1 p24 Gag, Vpr and Tat. These authors demonstrated that the use of overlapping peptides based on HIV-1 clade B consensus sequence was associated with weaker responses especially in the more variable Tat and Vpr regions (7).

1.5.2 The escape phenomenon

It is critical to know how sensitive T cell clones are to virus variability. The idea that T cells can cross-react broadly and that matching a vaccine to the virus subtype is not needed is not applicable in HIV-1 infections. Data indicate that most sequence variation in an epitope generate escape mutants (144). Moreover, viral escape from CTL-mediated immunity occurs frequently in HIV-1 as well as SIV infections (3, 4, 42, 68, 73, 80, 89, 92, 122, 134, 198) and therefore can lead to loss of vaccine-induced protection (18). Therefore, an ideal HIV-1 vaccine design would have a genetic sequence matching the virus in the population to be vaccinated (64). At a first glance, this would mean matching vaccine to clades. However, due to the differences within clades, the latter could lead to evasion of virus from induced T cell immunity. Thus, in addition to matching the vaccine and the clade, vaccinologists need to induce broad T cell responses directed against multiple epitopes in order to reduce the risk of escape.

1.5.2.1 Immunodominant versus subdominant epitopes

Immunodominance is another issue to consider in the design of a vaccine against HIV-1 (89, 286). In this case, T cell responses are predicted by the HLA type of the infected individual and thus exerting more pressure for virus escape. The selection of epitopes during antigen processing, different affinity of different epitopes on HLA molecules as well as half-life at the

cell surface are all possible mechanisms of immunodominance (283). Recent reports demonstrated the importance of subdominant epitopes in the control of HIV-1 replication (76). High allele frequencies were found to eliminate subdominant epitopes despite being effective at stimulating CTL responses. Moreover, high affinity CTL down-modulate peptide-MHC complexes on antigen-presenting cells leading to preferential expansion of high-affinity CTL (130, 204). Subsequently, preexisting CTL responses decrease CTL-specific responses to other epitopes within the construct. This highlights the negative impact that could be exerted by the use of centralized sequences in the face of immune escape due to preferential expansion of immunodominant CTL epitopes while ignoring subdominant ones. Thus expansion of specific CTL responses for multiple epitopes should be considered in designing HIV-1 vaccines.

Importantly, the immune system can develop novel $CD8^+$ T cell responses against CTL escape variants during chronic HIV-1 infection (6). Authors followed a number of HIV-1-1 infected individuals for more than 6 years following acute infection, 4 of which had an escape mutation in HLA-A*11 restricted Gag epitope. A decline in $CD8^+$ specific T cell responses was observed in these patients. However, following this event, a new $CD8^+$ T cell response was documented. This response to the escape variant was as strong as the one observed to the wild-type sequence. This finding supports the use of sequences encoding potential circulating variants leading to protection against escape mutation. *In vitro* studies showed that the virus-specific $CD8^+$ T cell responses directed against consensus sequences persist from earlier responses but do not recognize autologous virus (145). Thus more evidence is provided towards the importance of using autologous virus sequences in the design of HIV-1 vaccines especially in the face of an escape mutation. Consequently, HIV-1-1 vaccine based on a single virus sequence, particularly immunodominant, will induce CTL responses that are too specific to provide protection against

the quasispecies of the variable HIV-1-1, hence limiting the ability of this vaccine to control the virus.

Moreover, many studies demonstrate that HIV-1-1 escapes rapidly from $CD8^+$ T cell mediated immune responses not only within targeted epitopes but also flanking epitopes (3, 32, 131). Hypervariability regions in HIV-1 are also associated with specific CTL epitopes in individuals with a specific major MHC class I haplotypes (175). There have been reports that some CTL escape mutation revert after transmission and subsequently remain useful for CTL based vaccines (3, 80, 147). These findings emphasize the importance of determining which immune responses remain relevant and which ones are lost through escape mutations for vaccine design and highlighting the importance of CTL in shaping the evolution of HIV-1 and SIV variations (80). Moreover, intrapatient evolution of HIV-1 caused by CTL escape is not necessarily reflected into HIV-1 evolution at the population level. To account for these complexities, a therapeutic vaccine should be tailored towards the autologous virus present in an individual at the time of immunotherapy and thus dominant and subdominant epitopes would be introduced.

1.6 DENDRITIC CELL: FUNCTIONS

Dendritic cells (DC) are the most potent antigen presenting cells and have been attractive candidates in the development of vaccines (14, 16). They are important players in innate as well as adaptive immune responses. They are present at low numbers in peripheral tissues where they capture antigen by phagocytosis, macropinocytosis or receptor-mediated endocytosis. DC process antigens and load peptides onto MHC molecules which are transported to the cell surface

for antigen presentation (141). DC maturation is induced by pathogens through their cell wall components (e.g. lipopolysaccharide), viral products (double stranded RNA), and inflammatory cytokines such as IL-1 or TNF-α. Complete DC maturation takes place through CD40/CD40L-dependent and independent interaction between DC and helper T lymphocytes (255). Maturation takes place during DC migration from the peripheral tissue to the lymphoid organs and it is associated with the loss of endocytic and/or phagocytic function, morphological changes, changes in MHC class II molecules and most importantly upregulation of expression of co-stimulatory molecules CD80, CD86 and CD40 as well as expression of CD83 (13). During maturation, DC downregulate the expression of chemokine receptors such as CCR1, CCR5 and CCR6, constitutively expressed on immature DC and upregulate CCR7 expression driving DC into the lymph node in response to chemokines such as MIP3β or 6kine (164, 231). Once DC mature, they synthesize cytokines required for the development of helper T cells and CTL; these cytokines include IL-12, IL-15 and IL-18. In addition, mature DC produce chemokines essential for attracting naïve and memory T cells.

1.6.1 Antigen processing and presentation by Dendritic Cells

Extracellular antigens are processed and presented by MHC class II molecules. The MHC II-peptide complexes as well as CD86 are translocated to cell surface to induce primary T cell expansion through interactions with $CD4^+$ T cells (115). As a result of this interaction, T cells differentiate into Thelper (Th) 1 and Th 2 cells producing IFN- γ and IL-4, respectively. $CD4^+$ T cells are essential in licensing the DC to activate $CD8^+$ T cells *via* CD40L-CD40 interactions (219, 236) and maintenance of $CD8^+$ T cell memory.

MHC class I molecules process antigens synthesized in the cytoplasmic compartment (224). DC are also capable of exogenous antigen cross-presentation onto MHC class I molecules (1, 99). This pertains to the proteins derived from immune complexes, inactivated microbes or other antigens synthesized in other cells and then cross to MHC of DC (63, 101). Endogenously acquired antigens, i.e. synthesized proteins in the cytoplasm, are cleaved by the proteasomes into peptides, transported to the endoplasmic reticulum (ER) *via* transporters for antigen presentation (TAP), loaded onto MHC class I molecules and then transferred to cell surface where these complexes are recognized by $CD8^+$ T cells. Following contact with T cells, DC produce IL-15 which leads to priming of $CD8^+$ T cells including expansion and generation of effector functions.

Given the role of DC in immune control and the determination of the type of T cell response , it is logical to use these cells as targets for clinical applications such as resistance to infections, tumors , immunodeficiency and most important in vaccines. The optimistic promise of DC-based vaccine does not mean overlooking the abilities of certain pathogens to evade the immune system and directly affect DC functions.

1.6.2 Dendritic Cells and HIV-1-1 infection

For a better understanding of DC-based vaccination against HIV-1 or SIV, we need to know the role of DC in the entry of these viruses, their trafficking in DC and transfer to T cells. DC express CD4, an HIV-1 receptor, as well as chemokine receptors acting as coreceptors for HIV-1 (281). Consequently, many studies initially analyzed whether DC constitute virus replication sites or act as vehicles to transport the virus from the mucosa to activated T cells in secondary lymphoid organs. It has been found that coculture of resting T cells with DC leads to HIV-1 replication (39, 74, 213, 278). Moreover, virus production *in vitro* was found within syncitia,

confirmed *in vivo* by the formation of HIV-1-expressing syncitia at the surface of tonsils and adenoids (77, 78). Using the SIV model, infected DC with virus were seen in the mucosa. Reports on DC involvement in virus spread to $CD4^+$ T cells followed by virus replication have been demonstrated (112, 113). Moreover, replication of HIV-1/SIV in DC has also been demonstrated *in vivo* (78, 112, 113) and *in vitro* (84, 251). Since DC acquire antigens from peripheral tissues and transport them to lymphoid tissues, HIV-1/SIV may gain access to $CD4^+$ T cells through this pathway (215).

1.6.2.1 *trans*- or *cis*-infection

DC, the most potent APC, act as traffic line for HIV-1-1. The fate of HIV-1-1 in DC has been a controversial topic. The attachment of HIV-1-1 to DC takes place through glycoprotein (gp) 120 on the virus and mannose specific C-type lectin receptors (CLRs) on DC. DC-SIGN (CD-209) is the major CLR on some myeloid-derived DC. Immature DC express high levels of CLRs including CD207 (Langerin) on epithelial langerhans cells and CD206 (mannose receptors) on dermal DC (259). *trans*-infection is the process by which HIV-1 is transferred from monocyte-derived DC to $CD4^+$ T cells and that DC are not productively infected (84). Others believe that DC get infected before viral transfer to $CD4^+$ T cells (267). This takes place through *cis* infection where HIV-1-1 gp120 binds to CD4 receptor and to either CCR5 (R5 strains of HIV-1-1) or CXCR4 (X4 strains of HIV-1-1). Immature monocyte-derived DC express CD4 as well as CCR5 but less amounts of CXCR4. Recently, monocyte-derived DC were reported to process and degrade virus in compartments lacking endolysosomal markers, a process suggested to take place in initial phase where virus is transferred to T cells and a second phase where residual virus generate productive infection in immature DC leading to transfer to T cells (260). This was confirmed by comparing live and AT-2 treated HIV-1/SIV. HIV-1-1 is cytopathic following

syncitia formation between DC and T cells. The above data highlight the fact that DC might play the role of a "Trojan horse" for transport of HIV-1-1 and SIV to T cells for replication (166, 214). Most important is the binding of DC-SIGN to HIV-1-1 and consequently promoting infectivity to T cells. Thus, DC are involved in the offense of HIV-1-1 as well as defense of the infected host (221).

In any case, whether DC are productively infected or not during transfer of virus to $CD4^+$ T cells, monocyte-derived DC isolated from HIV-1-infected individuals at different stages of the disease (i.e. immunocompetent, immunosuppressed before initiation of treatment, as well as those receiving treatment with undetectable viral load) were recently found to express normal phenotype as well as allogenic stimulatory capacity as normal DC. Moreover, impaired antigen-specific $CD4^+$ T cell responses associated with HIV-1-1 infection was partially restored by antigen presentation of DC (193). Thus, more evidence is provided supporting the efficiency of DC in antigen presentation. As a result, immunization strategies against HIV-1 targeting DC offer a great advantage in the ability to stimulate antigen-specific protective immune responses (246).

1.6.3 Methods of antigen delivery to Dendritic Cells

It is well known that the method of antigen delivery to and uptake by DC affect the ability and efficiency of antigen presentation to T cells. The MHC class I pathway involves proteins generated within the cells that are cleaved by proteasomes and enzymes in the cytosol and thus stimulate $CD8^+$ T cells. Extracellular proteins endocytosed and processed in the lysosomal pathway will be presented by class II pathway leading to $CD4^+$ T cell stimulation (141). In addition, DC have the capacity to internalize exogenous antigens for CTL stimulation by cross-

priming. Thus, DC act as natural adjuvants. Several methods of *in vitro* antigen delivery to DC have been described. These include loading with peptides (86, 191, 237), proteins (114), viral vectors (94, 36, 71, 247, 292), and inactivated viral particles (156, 158), DNA based immunizations (53) and transfection of DC with RNA or plasmid DNA (86).

Although synthetic peptides are readily available for clinical use, the use of DC with peptides depends on the individual's HLA haplotype. The latter determines the MHC molecules expressed in an individual since different molecules bind and present different sets of peptides. Moreover, the peptide-MHC complexes have limited persistence on DC. Engulfed proteins are processed to peptides by the lysosomal/endosomal MHC class II pathway and thus induce $CD4^+$ T cell response only. DC pulsed with viral vectors or inactivated viral particles are effective at inducing HIV-1-specific T cell responses *in vitro*. Antigen specific T cell responses are induced in macaques vaccinated with DC expressing SIV or HIV-1 antigens post challenge with pathogenic virus strains (35, 155, 158, 191, 273). Viral vector pulsing has been based on the use of adenovirus, poxviruses, and lentivirus. These are preferred candidate for introducing antigen into DC due to high level transgene expression; however, their usefulness is limited due to undesired activation of DC as well as anti-vector responses due to preexisting immunity (36, 72, 118, 176, 268).

1.6.3.1 Non-viral gene transfer

A number of recent studies aimed at solving the above obstacles by using non-viral gene transfer methods. These methods consist of loading DC with DNA or mRNA (86). Poor expression of transgene combined with the toxicity caused by DNA transfection has limited the use of DNA (12, 146, 248, 261, 262, 268). Methods to enhance gene expression following transfection of DC with DNA have been proposed based on DC maturation (142). The use of DC transfected with

mRNA encoding tumor or virus antigen has been popular for the generation of high levels of gene expression associated with limited toxicity as compared to DNA and importantly the induction of antigen-specific T cell responses (86, 103, 104, 129, 169, 212, 248, 250, 266, 269, 279). mRNA transfection of DC was recently found to result in similar numbers of transducible cells as when using virus-mediated transfer (160).

It is clear that mRNA gained a wide acceptance as a valuable mean for gene transfer to DC; however, the debate still exists on the method to be used to attain best transfection efficiency results combined with sustained antigen production. Several methods have been described in the literature with variable results. Passive pulsing, electroporation, lipofection and recently nucleofection, a modified version of electroporation, have been used for transfection of DC with mRNA encoding tumor ad viral antigens (86, 129, 140, 146, 169, 266, 269, 279). Many lessons are learned from the use of these different methods to target tumor or viral antigen to DC for vaccination. The choice of antigen, the cytotoxicity of the method applied, the level of protein expression and its sustainability, the effect of transfection on the stimulatory capacity and cytokine production profile of transfected-DC are to be considered carefully when introducing antigen to DC.

1.7 HIV-1 VACCINES

1.7.1 First generation vaccines: stimulation of antibody responses

Soon after HIV-1 was identified, efforts to develop an effective vaccine began. The first vaccine candidates against HIV-1 were based on the use of recombinant envelope proteins in order to stimulate the neutralizing antibody responses similar to the successful generation of hepatitis B vaccine (121). These vaccines protected chimpanzees from HIV-1 infection but failed to protect healthy individuals (52) due to evasion mechanisms evolved by the virus (277). Consequently, it was concluded that the stimulation of antibodies to neutralize primary HIV-1 isolates is difficult (38).

1.7.2 Vaccines stimulating T cell responses

Hence, increased attention shifted to T cell mediated immunity especially due to its impact on the control of HIV-1 infection. Many studies support the role of T cell immunity in early and subsequent control of HIV-1 infection in humans as well as SIV infection in non-human primates. Consequently, the new generation of vaccines aimed at stimulating strong cell mediated immune responses. Many vaccine candidates developed and have been tested in the non-human primate model and some of which have entered human clinical trials. The use of animal models of HIV-1 infection proved to be valuable to determine the effect of suggested

vaccines on viral infection and disease progress. These approaches have been extensively reviewed and constitute of live attenuated, whole inactivated vaccines, recombinant proteins, synthetic peptides or lipopeptides, virus-like particles, DNA vaccines, live recombinant viral or bacterial vectored vaccines and finally DC loaded with inactivated virus or viral antigens (67, 167).

The pathogenically attenuated viruses providing safe and effective production against smallpox, measles and polio, have also protected against SIV infection in macaques (58); however, adult monkeys infected with these viruses developed a late onset disease and new born monkeys developed disease following infection. Similar results were obtained in humans as well. Thus the safety of this strategy is questionable (143). Historically, physically or chemically inactivated viruses provided effective immune responses against influenza and polio (148). Similarly, formalin-inactivated viruses have protected monkeys from SIV albeit for a short period of time (180); thus this approach did not offer a lot of optimism. An attractive source of HIV-1 antigens *in vivo* may be the live and apoptotic infected $CD4^+$ T cells. The latter was found to be more efficient at stimulating T cells as compared to direct infection or defective virus particles or proteins (162). Other vaccines include recombinant modified vaccinia virus (MVA), recombinant adenovirus-5, and recombinant vesicular stomatitis virus. These vaccines were tested in the monkey model. The challenge virus was an aggressive simian-human immunodeficiency virus (SHIV-1) 89.6P expressing CXCR4-specific HIV-1 envelope and leading to rapid decline of $CD4^+$ T cells and immunodeficiency (10, 20, 222, 241). In these studies, authors reported that animals remained healthy and did not lose $CD4^+$ T cells with no sterilizing immunity. In addition to the MVA model in non-human primates (108), the vaccination of macaques with a recombinant canarypox virus expressing SIV Gag, Pol and Env,

was found to be immunogenic after producing sufficient protein. The low frequency CTL responses induced post challenge was of short duration (233). The recombinant adenovirus vectors have been an attractive approach in the monkey model (21) and many candidates are entering the clinical trial phase (121, 167) and human volunteers were able to elicit HIV-1-specific T cell responses. One disadvantage associated with this strategy is the preexisting immunity to the vector leading to decline in immunogenicity (19). This problem is attempted to be resolved by plasmid DNA vaccination followed by recombinant adenovirus boosting. Moreover, Peptide-based vaccines were also found to induce protection in macaques infected with SHIV-1 (89.6P) (190, 191). Despite the immunogenicity of many of these models in infected animals and their ability to control the infecting virus, it is unknown how close the $CD8^+$ T cell responses in monkeys would be in humans (167) and how relevant the generated immune responses are in the face of virus escape.

1.7.3 Virus-like particles

The safety issues associated with the use of live attenuated vaccines especially in immunocompromised patients as well as the failure of recombinant gp120 to protect against HIV-1-1 and to generate neutralizing antibodies (38, 52) lead to the emergence of the idea of virus like particles (VLP) where the envelope is presented in its original form (66). These particles cannot replicate, nor produce virus and thus are safe. It has been recently demonstrated that SHIV-1 VLP bind to DC, are internalized and lead to significant up-regulation of CD83 and CD40 and subsequent release of IL-12, IFN-γ, and TNF-α. The immunogenicity of VLP is enhanced by loading them into DC where studies reported that HIV-1-1 VLP induced maturation of DC *in vitro* and *in vivo* (230). Although several advantages are associated with the use of

VLPs, it has been suggested since they have a similar structure as the wild-type viral structure, that they might induce a similar non protective effect. Moreover, VLPs are also considered to be less potent than live attenuated viruses or replicating viral vector-mediated vaccines since they are non-replicating.

1.7.4 Clinical trials

To date there is no approved vaccine against HIV-1-1 despite the effort and the numerous strategies devised for this purpose. Several approaches are in various stages in clinical trials with at least 5 completed but results not yet reported (167). These include recombinant DNA plasmids (70) , recombinant viral vectors such as the canarypox immunogens (ALVAC) (62), DNA prime and modified vaccinia Ankara (MVA) (181) (similar studies were performed earlier in nonhuman primates (10, 24) and lead to protection against disease), replication incompetent adenovirus vectors (241), sindbis virus-based replicon particles (264). VLPs, proteins as well as other candidates are being tested as well in clinical trials with a variety of HIV-1 immunogens in different geographic areas (121, 167).

There is a massive number of studies in progress in an effort to generate an effective HIV-1 vaccine. It is disappointing though that in most cases only $CD8^+$ T cell responses are targeted and the issues of virus variability as well as arising escape mutations during the course of the infection are not yet resolved.

1.8 DENDRITIC CELL-BASED VACCINES

DC have been extensively exploited for the generation of cancer as well as HIV-1 vaccines (86, 275) especially due to their role as potent antigen-presenting cells. Importantly, monocyte-derived DC isolated from HIV-1-infected individuals exhibit normal phenotype and cytokine production (47). In the HIV-1/SIV field, DC transduced with Gag protein were found to induce antigen-specific T cell responses *in vitro* (155). Moreover, when rhesus macaques infected with SIV and later patients chronically infected with HIV-1 were vaccinated with DC pulsed with heat-inactivated or chemically-inactivated HIV-1 or SIV, strong T cell responses were observed associated with a decrease in viral titers (81, 156 - 158). These DC-based prophylactic vaccine approaches against HIV-1-1 including vaccination of macaques with DC expressing SIV or HIV-1 antigens (35, 190, 273) have been based on well characterized strains of virus that may not be suitable for therapeutic vaccination as epitopes expressed by DC may not represent those in the infected individual. Efforts to include autologous viral Ag in therapeutic vaccines have relied on inactivated virus particles propagated from individual patients (157); however, this procedure is labor-intensive and may not uniformly lead to sufficient virus recovery for DC loading in all patients.

1.8.1 DNA versus mRNA

Recently more focus has been directed towards plasmid DNA and RNA based DC vaccination (40, 86, 97, 98, 212, 261). Non-viral gene delivery methods are more appealing than viral vector-based strategies due to safety issues. Consequently, the use of nucleic acids was studied in cancer as well as viral immunotherapies. DNA and mRNA are both safe and easily produced and lead to

protein translation in the cytoplasm of DC. In addition, both nucleic acids can encode for multiple epitopes and do not require the knowledge of the HLA haplotype of each patient. DNA has been reported to be not suitable for transfection of DC (160, 248, 269). Major advantages of RNA transfection over DNA is safety where the half-life of stable mRNA is less than 24 hours in mammalian cells whereas unintegrated DNA can persist in non-dividing cells (40, 174). Limited DNA has been reported to undergo trafficking to the nucleus (161). In contrast, mRNA is immediately translated in the cytoplasm and there is no danger of integration in cellular genetic material as the case with DNA. Thus mRNA-based DC therapies have been more popular in patients with cancer (15, 86, 191, 237) where strong antitumor responses have been observed against prostate-specific antigen (PSA) (104), carcinoembryonic antigen (CEA) (185, 179), and telomerase reversed transcriptase (TERT) (251) following transfection of DC with the respective mRNA. As a result of the successful *in vitro* work, the use of mRNA-transfected DC is being translated into phase I/II prostate cancer and renal cancer clinical trial (86, 96, 103, 212).

The use of DC transfected with mRNA encoding tumor antigens has been generally successful. The efficient handling and presentation of antigen by DC make these cells most appropriate for the development of HIV-1 vaccines stimulating robust T cell-mediated immunity. Importantly, we (169) and others (129, 266, 279) demonstrated that DC transfected with mRNA encoding SIV or HIV-1 antigen resulted in strong primary $CD4^{+}$ and $CD8^{+}$ T cell immune responses *in vitro*. Similarly, polyclonal T cell responses were induced *in vitro* against DC transfected with CMV RNA (102) and human papillomavirus RNA (257).

The best way to incorporate HIV-1 evolutionary path into a vaccine approach is to use autologous virus antigen, specifically mRNA-encoded antigen. Autologous virus will contain multiple epitopes derived from the circulating strain during infection. The inclusion of dominant

and subdominant epitopes demonstrated to be useful for protection especially after emergence of escape variants will be included in this design. In addition, when using autologous forms of antigen, we don't need to worry about identifying immunodominant CTL epitopes across HIV-1 genome and consequently clade specificity versus cross-clade reactivity for the vaccine design especially since data show significant differences in CTL epitopes within Gag, Rev and Tat clustering in different ethnic groups infected by different HIV-1-subtypes (195).

1.8.2 Nucleic acid based-DC vaccination for stimulation of $CD4^+$ and $CD8^+$ T cell responses

Once mRNA or DNA is translated into proteins in DC, they will be degraded into peptides presented to MHC class I. Given the need to promote both $CD4^+$ and $CD8^+$ T cell responses in therapeutic immunization strategies, antigens have been modified to generate better MHC class I and MHC class II presentation. This could be achieved by using nucleic acids bearing lysosomal-associated membrane protein-1 (LAMP-1) targeting signal for the channeling of endogenously expressed proteins in DC into the endosomal/lysosomal compartment. LAMP-1 is a type 1 transmembrane protein mainly localized to lysosomes and endosomes (282). A number of antigens have been reported to stimulate enhanced immune responses when targeted to the lysosomal membrane as compared with vaccines encoding native antigens: vaccinia vectors HIV-1-1 gp160/LAMP (226, 228), human papilloma virus E7/LAMP (282), and cytomegalovirus pp65/LAMP (29). Plasmid DNA antigen/LAMP vaccines encoding dengue virus 2 premembrane /envelope /LAMP (217) as well as human papilloma virus E7/LAMP (119) were also reported to induce better $CD4^+$ T cell responses. DC transfected with mRNA bearing LAMP and encoding carcinoma antigen (185) and telomerase reverse transcriptase have also

been documented in the cancer field (251). Recently, DC transfected with mRNA encoding hTERT/LAMP-1 induced a strong $CD4^+$ and $CD8^+$ T cell responses in patients with prostate cancer (85). Similarly, HIV-1-1 p55Gag encoded in LAMP-1 as a DNA plasmid vaccine chimera elicited enhanced cellular as well as humoral immune responses in immunized mice with a 10-fold increase in $CD4^+$ T cell responses and 4- to 5-fold increase in $CD8^+$ T cell responses (61, 163). In the SIV/HIV-1 field (129, 169), it has been recently demonstrated that monocyte-derived DC transfected with mRNA encoding SIV Gag and HIV-1 Nef bearing LAMP-1, respectively, were able to induce $CD8^+$as well as $CD4^+$ antigen-specific T cell responses *in vitro*.

1.9 CONCLUDING REMARKS

During the past few years, significant progress in the study of DC biology as well as various transfection methods devised for antigen loading of DC facilitated the use of these sentinels for the generation of tumor and viral vaccines. In all the studies briefly reviewed above, it is clear that DC are the decision makers regarding the type and the quantity of T cell immunity. The generated antigen-specific T cells should be able of cytokine production, proliferation upon reexposure to antigen, migration to the site of injury and CTL function. Moreover, $CD4^+$ T cells have to be taken into account for optimal vaccine specific immunity due to their role in maintenance of $CD8^+$ T cell memory. The role of DC along with antigens and T cells constitute the core of immune control. The primary goal of an AIDS vaccine is to prevent establishment of a persistent infection and consequently delay progression to disease through the development of strong cellular responses. The development of HIV-1/AIDS vaccine deals with two major problems: the genetic variability of HIV-1 as well as its propensity for immune evasion. These issues need to be addressed in the design of an immunogen. Our work is based on the use of mRNA encoding autologous virus sequences isolated from SIV-infected macaques to load DC for the generation of relevant and potent cell mediated immunity, and thus accounting for the complexities associated with HIV-1 infection. mRNA-based DC vaccines are safe, easy and clinically applicable. In conclusion, transfection of DC with mRNA offers practical as well as immunological advantages over other methods for therapeutic immunization against HIV-1.

2.0 HYPOTHESIS AND SPECIFIC AIMS

An important challenge in the development of vaccines for HIV-1 is the diversity of clinical isolates coupled with the ability of the virus to undergo escape mutations in T cell epitopes through selective pressure *in vivo*. Hence, CTL responses induced in response to the infecting strain might be incapable of controlling circulating virus during the course of infection. Thus, a therapeutic vaccine against HIV-1 should be tailored towards the autologous virus present in an individual at the time of immunotherapy. We hypothesize that therapeutic immunization strategies aiming at optimizing antigen presentation and stimulation of potent T cell responses could be achieved by loading DC from SIV infected macaques with mRNA encoding autologous virus sequences isolated during the course of infection**.** We will use the rhesus macaque SIV model since it provides an ideal preclinical setting to test the therapeutic potential of DC-based vaccines using virus-derived mRNA. **The specific aims of this project are**:

1) **To compare the transfection efficiency of monocyte-derived DC transfected with plasmid DNA or *in vitro* transcribed mRNA.** In this aim, a side-by-side comparison of GFP expression post-transfection of K562 cells, human and monkey monocyte-derived DC with plasmid DNA or mRNA will be performed using four different transfection methods. Depending on the results of these preliminary experiments, we will adopt a transfection strategy as a method of choice. We will characterize the phenotype of

monocyte-derived DC following transfection with pEGFP-N1 DNA and *gfp* mRNA. Time course kinetics will also be performed post transfection of monocyte-derived DC with DNA or mRNA to determine the durability of gene expression. Finally, a comparison of the effect of DC transfected with DNA or mRNA encoding wild-type SIV Gag on the generation of antigen-specific T cell responses will be investigated.

2) **To test the ability of DC transfected with virus-derived mRNA to stimulate specific T cell responses *in vitro* in an entirely autologous system.** In this aim, we will assess the utility of mRNA-based DC transfection in the SIV model. The ability of *gag* mRNA to stimulate Gag-specific T cell responses will be assessed first in a heterologous system. Afterwards, DC will be nucleofected with *gag* mRNA isolated during infection and the ability to stimulate autologous T cell responses will be determined. In addition, the impact of a CTL escape mutation in an immunodominant epitope (CM9) on T cell responses will also be assessed. Finally, we will determine the efficacy of *gag* mRNA-transfected DC bearing the lysosomal-associated membrane protein-1 (LAMP-1) targeting signal to induce $CD4^+$ and $CD8^+$ T cell responses in the presence and absence of a CTL escape mutation.

3) **To test the ability of costimulatory TNFR family molecules CD134 (OX40L), and CD70 to improve the immunostimulatory capacity of monkey monocyte-derived DC.** We will first determine the endogenous expression of these molecules by SIV-naïve and SIV-infected DC. Following generation of mRNA encoding monkey CD70 or OX40L, we will test the immunostimulatory capacity of DC transfected with *gag* mRNA and cotransfected with CD70 mRNA, OX40L or both.

3.0 CHAPTER ONE. HIGH-LEVEL ANTIGEN EXPRESSION AND SUSTAINED ANTIGEN PRESENTATION IN DENDRITIC CELLS NUCLEOFECTED WITH MRNA BUT NOT DNA

Nada M. Melhem,[1,2] Sherrianne M. Gleason,[1,2] Xiang Dong Liu,[1,2] and Simon M. Barratt-Boyes[1,2,3]

This chapter is adapted from a study submitted to a peer-reviwed journal for publication.

Running title: mRNA and DNA nucleofection of dendritic cells

[1] Center for Vaccine Research and [2]Departments of Infectious Diseases and Microbiology and [3]Immunology, University of Pittsburgh, Pittsburgh, Pennsylvania, 15261, USA

Correspondence: Simon M. Barratt-Boyes, 9046 BST3, University of Pittsburgh, 3501 Fifth Avenue, Pittsburgh, Pennsylvania 15261, USA. Phone: (412) 383-7537. Fax: (412) 624-4577. E-mail: smbb@pitt.edu.

Keywords: Human, nonhuman primate, transfection, T cell stimulation, immunotherapy

Word count: Approximately 8,400 words

3.1 PREFACE

This chapter is in partial fulfillment of specific aim 1. Nada M. Melhem wrote the manuscript as well as ran all the experiments except for the confocal microscopy images performed by Sherrianne M. Gleason and the generation of plamsids done by Xiang Dong Liu. Simon M. Barratt-Boyes provided guidance and financial support.

3.2 ABSTRACT

Nucleofection is an effective means of delivering transgenes to primary cell lines but its use in introducing DNA or mRNA into dendritic cells (DC) has not been widely investigated. We show that nucleofection is a superior means of transfecting human and monkey monocyte-derived DC with DNA and mRNA as compared to lipofection and conventional electroporation. However, delivery of DNA and mRNA had significantly different outcomes in transfected DC. DC nucleofected with DNA encoding the GFP gene had poor Ag expression and viability and were refractory to maturation with CD40 ligand (CD40L). In contrast, >90% of DC nucleofected with mRNA expressed uniform and high GFP levels from 3 h to 96 h post transfection and had a normal maturation response to CD40 ligation. Monkey DC nucleofected with mRNA encoding wild type simian immunodeficiency virus (SIV) Gag stimulated robust Ag-specific effector T cell responses in autologous PBMC from a vaccinated monkey both at 24 h and 48 h post nucleofection, whereas no detectable T cell response was noted when DC were nucleofected with DNA encoding the same Gag sequence. These data indicate that mRNA nucleofection could be an optimal means of transfecting DC with tumor or viral Ag for DC-based immunotherapy.

3.3 INTRODUCTION

Monocyte-derived dendritic cells (DC) are currently being used as therapeutic vaccines for cancer and infectious diseases and approaches to express tumor-associated or viral Ag in DC are being widely sought (15, 86, 157, 191, 212). Viral vectors based on adenovirus, poxviruses and lentivirus induce high level Ag expression in DC but may be associated with safety concerns or alteration of DC function, limiting their usefulness (69, 72, 118, 176, 268). An attractive alternative to vector-mediated delivery of Ag into DC is the use of nonviral gene transfer based on DNA or mRNA. DNA transfection of DC has been used successfully in some studies but is limited by poor expression levels and toxicity (12, 146, 248, 262, 268) although methods to enhance expression based on DC maturation have been proposed (142). mRNA transfection of DC is being increasingly utilized for cancer immunotherapy and *in vitro* stimulation of virus-specific T cells but approaches to deliver mRNA into primary DC cultures have been limited (86, 103, 129, 212, 229, 250, 266, 269).

Recently, nucleofection has emerged as a superior method for delivery of transgenes to primary cell lines including cytokine-induced killer cells, neurons, keratinocytes, macrophages and DC (56, 82, 116, 140, 146, 169, 182, 256). Nucleofection has primarily been used as a method of introducing DNA into cells as it is reported to deliver DNA directly into the nucleus, enhancing gene expression (95). However, recent studies suggest that nucleofection of DC with mRNA is an effective means of inducing high level Ag expression (140, 169). While conventional methods of gene delivery have been compared using monocyte-derived DC (248, 278), a limited number of studies have employed the more efficient process of nucleofection to evaluate DNA and mRNA delivery into these cells (140), and none to our knowledge have

compared the capacity for mRNA- and DNA-nucleofected DC to stimulate Ag-specific T cell responses.

In the present study we did a comprehensive analysis of DNA and mRNA transfection of human and monkey monocyte-derived DC, comparing liposomal transfection methods and conventional electroporation with nucleofection. We confirm that nucleofection is a superior method for delivery of both DNA and mRNA into primary DC lines. DC nucleofected with mRNA had rapid and sustained Ag expression with limited toxicity, were responsive to maturation stimuli and induced robust virus-specific effector T cell responses. In contrast, DC nucleofected with DNA had limited Ag expression and poor viability, were refractory to maturation with CD40L, and were unable to stimulate detectable T cell responses to viral Ag. These results indicate that nucleofection of DC with mRNA is preferred for immunotherapeutic applications, whereas nucleofection with DNA may result in ineffective DC.

3.4 MATERIALS AND METHODS

3.4.1 Plasmid and mRNA generation

Generation of the pSP73/GFP/A64 and pSP73/SIVmac239Gag/A64 plasmids and *in vitro* transcription of mRNA were performed as described previously (169). pEGFP-N1 DNA was a gift from Dr. Velpandi Ayyavoo, University of Pittsburgh. pSP73/SIVmac239Gag/A64 was used as a template for the amplification of Gag by PCR using the following forward and reverse primers, respectively: pGagN1F:5'GCGCTCGAGGCCACCATGGGCGTGAG3'; pGagN1R:5' CGCGCGGCCGCTTACTTGCCCAACTGCATGTAG 3'. pEGFP-N1 DNA was digested with XhoI and NotI and the larger vector band retrieved by gel purification. XhoI- and NotI-digested gag PCR product was inserted to the XhoI- and NotI- digested pEGFP-N1 DNA to generate pSIVmac239Gag-N1.

3.4.2 Cells

DC were cultured from purified blood monocytes of SIV-naïve rhesus macaques or healthy human volunteers as described (22). In some experiments DC were matured for 24 h or 48 h with 3 μg/ml recombinant trimeric CD40L (Immunex, Seattle, WA) as described (22). Approval was obtained from the institutional review board prior to experiments involving human samples and from the institutional animal use and care committee for all experiments involving rhesus macaque samples. K562 cells were grown in Iscove's Modified Dulbecco's medium (Hyclone, Logan, UT) supplemented with 10% FBS.

3.4.3 Transfection of K562 cells and DC

K562 cells were washed twice with Opti-MEM (Gibco Invitrogen Corporation, Frederick, MD) and transfected with TransFast transfection reagent (Promega, Madison, WI) or Transmessenger reagent (Qiagen, Valencia, CA) by adding 2 or 4 μg GFP mRNA or DNA in Opti-MEM with other reagents provided by the manufacturers to 1 x 10^6 cells as described (169). For electroporation, 1 x 10^6 K562 cells were electroporated with 10 μg mRNA or DNA in a total volume of 250μl in a 0.4-cm cuvette using the Gene Pulser II Electroporation System (Bio-Rad, Hercules, CA) with voltage/capacitance settings of 260 V/150 μF or 300 V/150 μF, respectively (269). Immediately after electroporation, cells were incubated for 5 min on ice. For nucleofection 10 μg DNA or mRNA was added to 1 x 10^6 K562 cells in Opti-MEM in a final volume of 100μl and transfected in a 2 mm-wide electroporation cuvette (BTX, San Diego, CA) using the T-16 program of the Amaxa nucleofector (Amaxa, Koln, Germany). Smaller amounts of mRNA and DNA were used for lipofection as compared to electroporation and nucleofection as toxicity was noted at higher amounts with the former methods (data not shown). K562 cells were cultured in Iscove's Modified Dulbecco's medium for 24 h at 37^{o}C following transfection. DC were transfected as immature cells (day 5 of culture) or as mature cells after 24 h incubation with CD40L (day 6 of culture). Lipofection using TransFast or TransMessenger reagents was done as for K562 cells. For electroporation, 1-2 x 10^6 DC in Opti-MEM were mixed with 5 ug to 20 μg mRNA or DNA depending on the experiment and electroporated as for K562 cells using voltage/capacitance settings of 300 V/150μF and 250 V/300μF, respectively, as described (169, 248). Nucleofection of DC was done as described for K562 cells using 5 ug to 20 μg mRNA or DNA and the U-02 or T-01 Amaxa program. Following transfection, DC were cultured in pre-

warm complete RPMI supplemented with GM-CSF and IL-4 for 24 h or 48 h at 37^{o}C, with and without CD40L, as described (169).

3.4.4 Confocal microscopy

DC nucleofected with pEGFP-N1 DNA or *gfp* mRNA 24 h previously were harvested using 20 mM EDTA and resuspended in PBS prior to settling onto glass slides at 37^{o}C for 1 h. Adhered cells were fixed with 2% paraformaldehyde for 15 min and washed in PBS. Gelvatol was used to apply coverslips to slides. DC were imaged for green fluorescent protein and differential interference contrast using an Olympus Fluoview 500 laser scanning confocal microscope (Olympus, Center Valley, PA). Images were collected using MetaMorph software (Molecular Devices, Sunnyvale, CA).

3.4.5 Flow cytometric analysis

GFP expression in transfected K562 cells and DC, and expression of HLA-DR, CD80, CD83, CD86 and CD40 on transfected DC were done as described (169).

3.4.6 ELISPOT assay

Gag-specific IFN-γ ELISPOT assays were done as described (169). Briefly, immature monkey DC were nucleofected with 10 ug pSIVmac239Gag-N1DNA or with *gag* mRNA transcribed from pSP73/SIVmac239Gag/A64 and simultaneously matured with CD40L (3 ug/ml) for 24 h or 48 h prior to incubation with autologous PBMC at a 1: 10 ratio. Control cells were nucleofected

with pEGFP-N1 DNA or *gfp* mRNA transcribed from pSP73/GFP/A64 or mock-transfected. IFN-γ spot-forming cells were developed and enumerated as described (21).

3.5 RESULTS

3.5.1 Nucleofection with mRNA is a superior method of introducing transgene into primary human and monkey monocyte-derived DC

We performed a side-by-side comparison of Transfast and Transmessenger lipofection methods along with electroporation and nucleofection using a GFP reporter construct. Cells were either transfected with pEGFP-N1 DNA or *in vitro*-transcribed *gfp* mRNA that was generated from the plasmid pSP73/GFP/A64 (169) and monitored for GFP expression at 24 h by flow cytometry. For lipofection methods we used 4 ug DNA or mRNA as toxicity was noted at higher amounts (data not shown), whereas for electroporation and nucleofection we used 10 ug DNA or mRNA. In initial experiments we used the chronic myelogenous leukemic cell line K562 that is readily transfected with plasmid DNA or mRNA (23, 269). Transfection of K562 cells with EGFP-N1 DNA using the two lipofection methods produced no detectable GFP fluorescence, whereas transfection *via* electroporation resulted in 16% GFP-expressing cells at 24 h. In contrast, 60% of K562 cells expressed GFP following nucleofection with EGFP-N1 DNA using the T-16 program of the nucleofector device, which was shown in preliminary experiments to generate the highest efficiency of transfection with this cell line (Figure 1A and data not shown). Transfection with mRNA was significantly more effective than transfection with DNA by all methods, with 8% to

16% GFP expression following lipofection, and 86% and 91% of K562 cells expressing GFP following electroporation and nucleofection, respectively (Figure 1A).

We next tested the capacity of lipofection, electroporation and nucleofection to transfect human and monkey immature monocyte-derived DC. In addition to measuring GFP expression at 24 h post transfection we assessed the effect of transfection on DC viability using the standard approach of trypan blue exclusion. Transfection of DC with DNA using lipofection or electroporation produced negligible GFP expression and significant cell death, with viability ranging from 40% to 57% (Figure 1B and C; Table 1). Nucleofection with DNA using the U-02 program was more effective than the other methods at generating GFP-expressing DC but was associated with enhanced cell death, with only 24% of DC remaining viable at 24 h post transfection (Figure 1B and C; Table 1), similar to other reports (146). Switching the nucleofector program to T-01 as favored by others (146) did not enhance transfection efficiency, and increasing the quantity of DNA to 20 ug was associated with a significant increase in toxicity (data not shown). Lipofection with mRNA resulted in variable transfection of DC, with maximum expression noted in monkey DC following Transmessenger lipofection, although the proportion of viable cells was only 52% to 55% (Figure 1B and C; Table 1). In contrast to K562 cells electroporation of DC with mRNA was inefficient, with expression levels not exceeding 17% and viability averaging only 46% (Figure 1B and C; Table 1). However, nucleofection with mRNA using the U-02 program was a highly effective means of transfecting human and monkey monocyte-derived DC, with the proportion of DC expressing GFP reaching 96% at 24 h post transfection (Figure 1B and C, bottom rows; Table 1). Nucleofection with mRNA maintained the highest cell viability of all methods, although the proportion of viable cells at 24 h post transfection was still only 68% (Table 1), consistent with other reports (140). Similar

transfection efficiency and DC viability were noted when nucleofection was performed with a range of mRNA from 5 ug to 20 ug (data not shown).

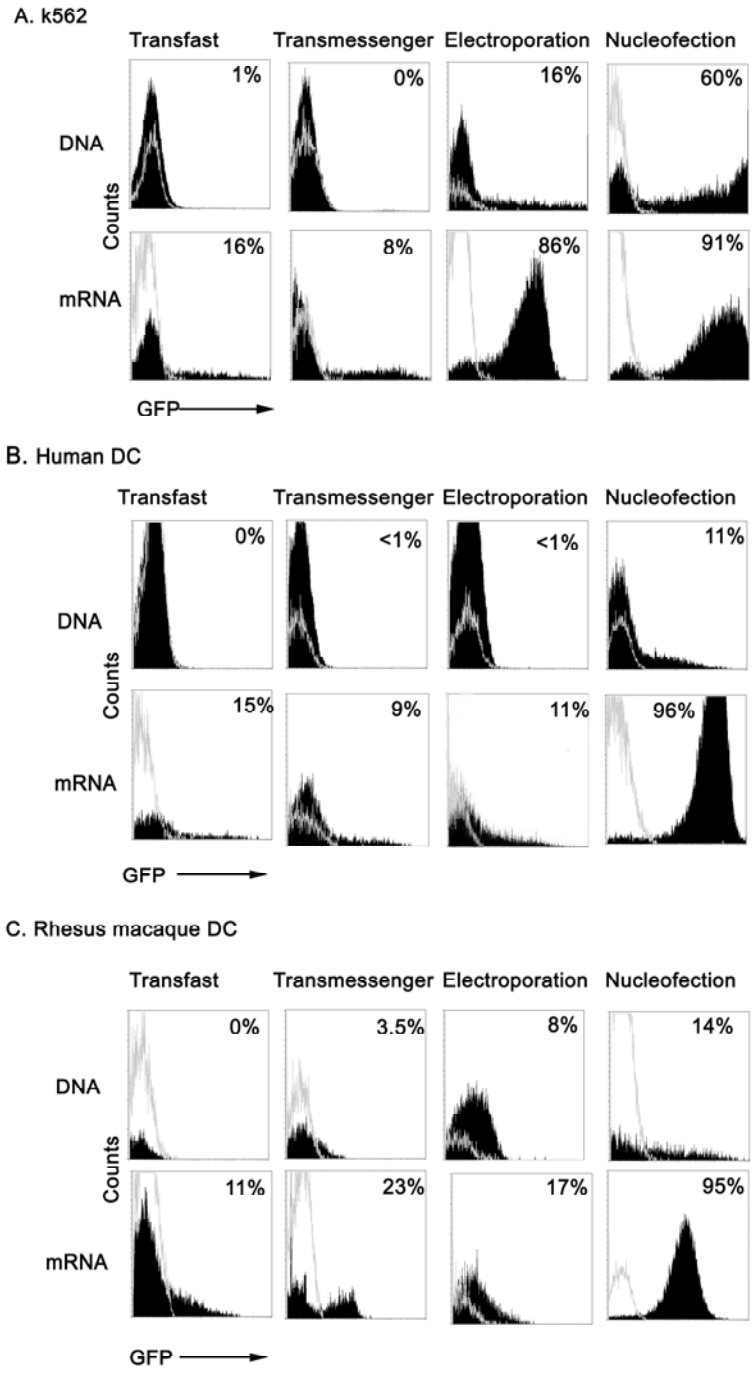

Figure 1. Evaluation of DNA and mRNA transfection of K562 cells and human and monkey DC. (A) K562 cells were transfected with pEGFP-N1 DNA (top row; filled histograms) or *gfp* mRNA (bottom row; filled histograms) or mock transfected (empty histograms) using the methods indicated and GFP expression determined 24 h later by flow cytometry. 4 ug DNA or mRNA was used for lipofection with Transfast and Transmessenger reagents, whereas 10 ug DNA or mRNA were used for electroporation and nucleofection. **(B, C)** Immature human **(B)** or rhesus macaque **(C)** monocyte-derived DC were transfected with GFP DNA or *gfp* mRNA and analyzed as for **(A)**. Numbers represent the percent of cells expressing GFP based on mock transfection. Results represent three different experiments.

Table 1. Gene expression and cell viability following transfection of immature monkey monocyte-derived DC

Method	*DNA*[a]		*RNA*[b]	
	GFP expression[c]	*Viability*[d]	*GFP expression*	*Viability*
Transfast[e]	**0**	**57 (3)**[f]	**7 (3)**	**52 (4)**
Transmessenger[e]	**2 (1)**	**49 (1)**	**14 (8)**	**55 (4)**
Electroporation[g]	**4 (7)**	**40 (2)**	**9 (7)**	**46 (3)**
Nucleofection[h]	**10 (4)**	**24 (5)**	**91 (3)**	**68 (6)**

[a]EGFP-N1 DNA

[b]In vitro-transcribed *gfp* mRNA

[c]Percentage of cells expressing GFP at 24 h post transfection

[d]Percent viable cells at 24 h post transfection

[e]4 ug DNA or mRNA was used with both lipofection methods

[f]Mean (SEM) of 2-5 experiments

[g]Cells were electroporated with 10 ug DNA or mRNA using 250V/300uF or 300V/150uF settings, respectively

[h]Amaxa U-02 program was used for both DNA and mRNA

Flow cytometric analysis of nucleofected cells indicated that GFP expression varied in intensity and uniformity depending on whether DNA or mRNA was used, with DNA producing a wide range of fluorescence and mRNA generating uniform expression in almost all cells (Figure 1B and C). To evaluate this in more detail we examined DC by confocal microscopy 24 h after DNA and mRNA nucleofection. The majority of mRNA-transfected DC showed a similar intensity of GFP expression when examined individually by microscopy, whereas GFP expression in DNA-transfected DC was highly variable; with some cells having intense fluorescence and a majority having weak or undetectable fluorescence (Figure 2). Expression of GFP in DNA- or mRNA-transfected DC was cytoplasmic in distribution, as expected (Figure 2). Taken together, these data indicate that nucleofection with mRNA is a superior method of introducing transgenes into DC both with respect to Ag expression and cell viability.

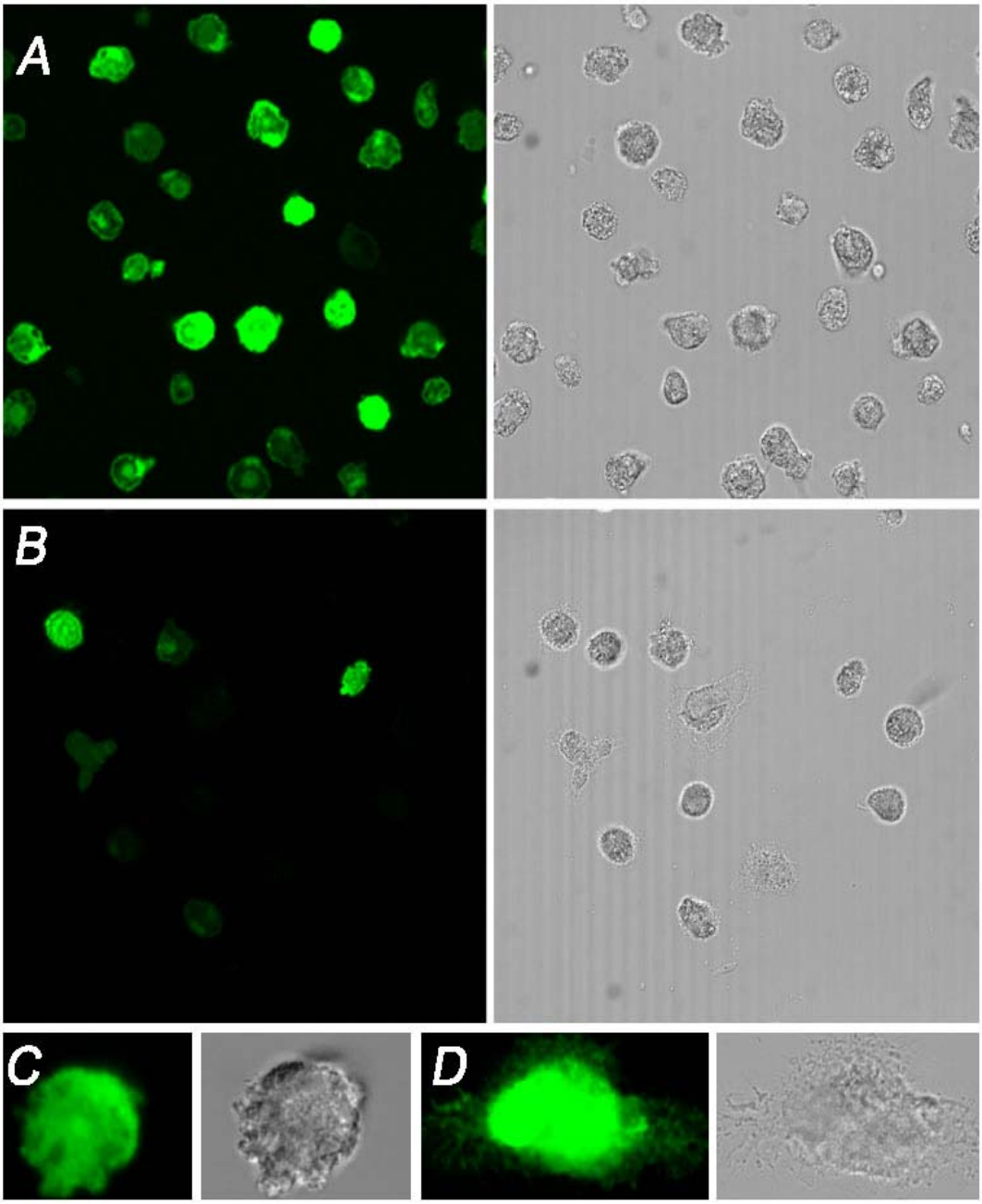

Figure 2. Expression of GFP in mRNA- and DNA-nucleofected DC. Immature monkey monocyte-derived DC were nucleofected with *gfp* mRNA (A, C) or pEGFP-N1 DNA (B, D) and examined by confocal microscopy 24 h later. Shown are GFP expression (left) and differential interference contrast (right). (A,B) Original magnification, X200. (C, D) Original magnification, X1500.

3.5.2 mRNA nucleofection of DC produces rapid and sustained expression of transgene

A potential limitation of mRNA transfection of DC for immunotherapy is that mRNA is labile in cells and may be degraded rapidly resulting in limited duration of Ag expression. We therefore assessed the durability of transgene expression in monkey immature monocyte-derived DC by harvesting cells at various intervals after DNA or mRNA transfection and determining the proportion of cells expressing GFP by flow cytometry. For these and all other experiments we focused on nucleofection as the preferred method of transfection. GFP expression following nucleofection of DC with pEGFP-N1 DNA was detectable at 3 h post transfection and maintained relatively constant levels of expression from 6 h to 48 h post transfection after which the proportion of GFP-expressing cells markedly declined (Figure 3), similar to other reports (146, 262). In contrast, the proportion of monkey DC expressing GFP following nucleofection with *in vitro*-transcribed *gfp* mRNA reached maximal levels by 3 h and stayed at this high level for 96 h, being the duration of the experiment (Figure 3). Similar rapid and sustained kinetics of transgene expression were noted in other studies following mRNA delivery to human DC using either electroporation or nucleofection (140, 169, 263, 269). These findings indicate that delivery of mRNA rather than DNA generates more durable expression of transgene in monocyte-derived DC.

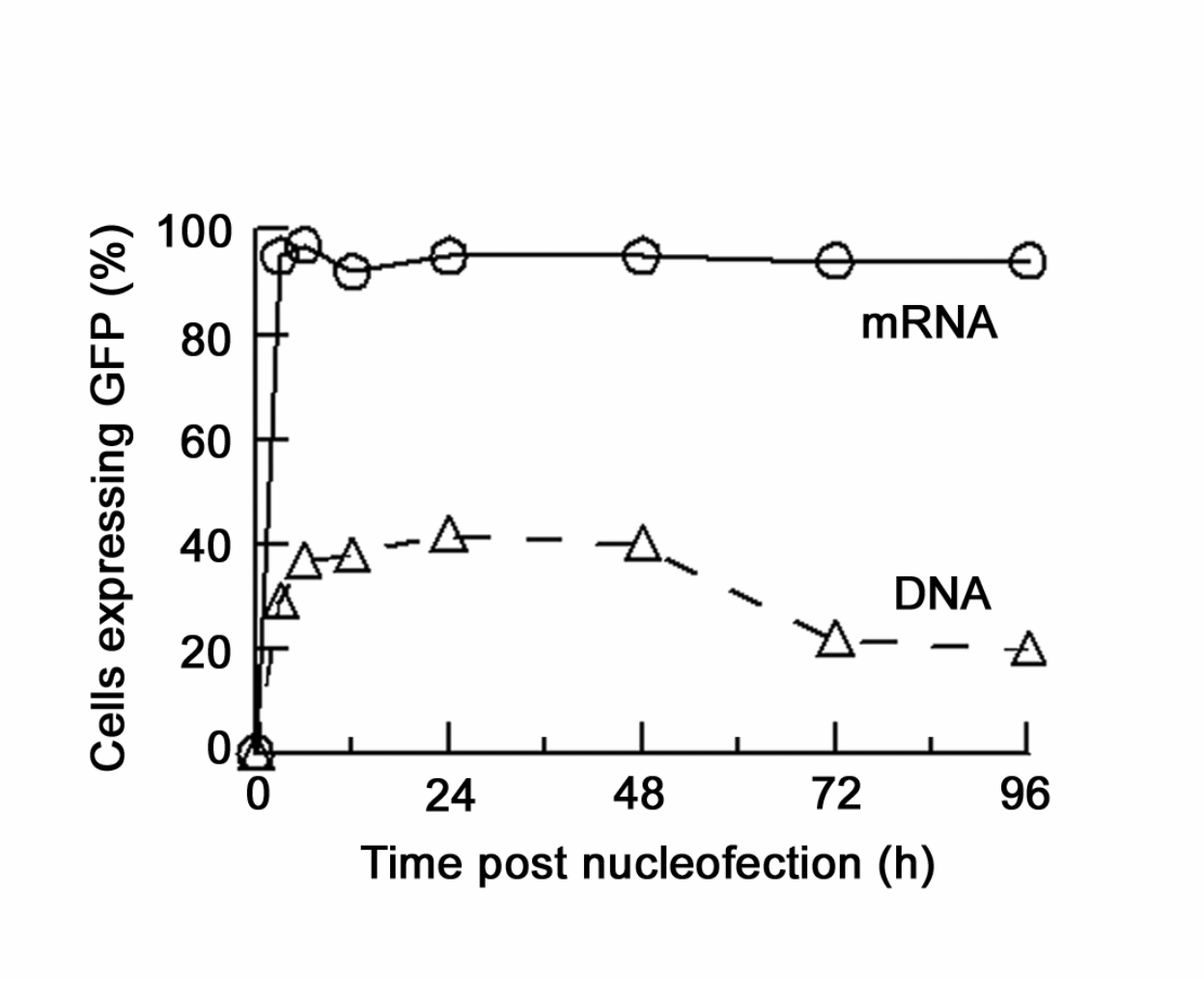

Figure 3. High-level and durable GFP expression in monkey DC nucleofected with *gfp* mRNA but not pEGFP-N1 DNA. Immature monkey monocyte-derived DC were nucleofected with 10µg *gfp* mRNA or pEGFP-N1 DNA and GFP expression determined at various intervals post transfection by flow cytometry.

3.5.3 Relationship between DNA and mRNA nucleofection and DC maturation

Immature DC are specialized in Ag uptake and as such transfection of DC with either DNA or mRNA is traditionally done at the immature stage of differentiation. However, it has been reported that DNA transfection is more effective in murine DC that have undergone spontaneous maturation in culture, and that transfection efficiency is markedly increased with concomitant CD40 ligation to induce terminal differentiation (142). To determine the influence of DC maturation on transfection efficiency we nucleofected human monocyte-derived DC with DNA and mRNA encoding *gfp* with and without prior treatment of cells with CD40 ligand (CD40L) for 24 h to induce maturation. Maturation was confirmed by phenotypic analysis using flow cytometry (data not shown). Prior DC maturation did not enhance GFP expression, as nucleofection of DC with DNA or mRNA generated similar levels of transgene expression regardless of the maturation state at the time of gene delivery (Figure 4A).

We next evaluated the responsiveness of DNA- and mRNA-nucleofected DC to maturation. Human immature DC were nucleofected with pEGFP-N1 DNA or *gfp* mRNA or mock nucleofected and either left untreated or treated immediately with CD40L. Cells were harvested and their phenotype analyzed 24 h post nucleofection by flow cytometry. To ensure that the phenotype of only transgene-expressing cells was analyzed, cells were gated based on GFP expression. Mock-nucleofected immature DC expressed relatively high levels of HLA-DR and CD40 and low levels of CD86 and lacked expression of CD80 and CD83, as expected (Figure 4B and C) (22). Nucleofection of immature DC with pEGFP-N1 DNA did not induce maturation based on phenotype (Figure 4B). However, DC nucleofected with DNA were refractory to CD40L, as GFP-expressing DC had negligible increases in CD83, CD86 and CD80 expression following CD40 ligation as compared to mock-nucleofected cells (Figure 4B). In contrast, immature DC

nucleofected with *gfp* mRNA had a minor shift in expression of CD83 and CD80 as compared to mock-nucleofected DC and responded normally to subsequent CD40 ligation, with increases in CD83, CD86 and CD80 expression similar to mock-nucleofected cells (Figure 4C). These data indicate that mRNA-nucleofected DC are responsive whereas DNA-nucleofected DC are refractory to maturation.

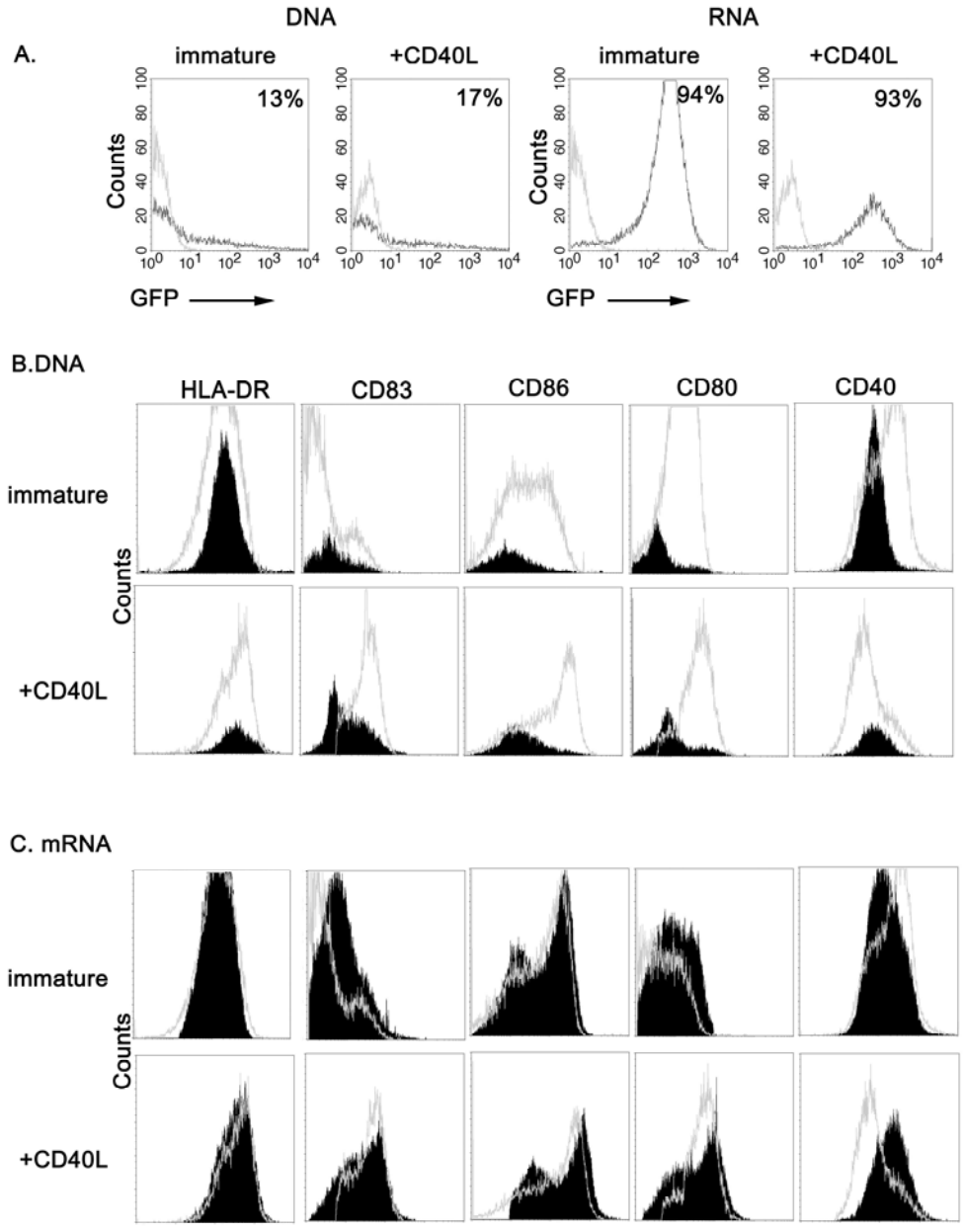

Figure 4. Maturation potential of mRNA-nucleofected or plasmid DNA-nucleofected human monocyte-derived DC. Maturation potential of mRNA-nucleofected or plasmid DNA-nucleofected human monocyte-derived DC. (A) Day 5 immature human monocyte-derived DC were nucleofected with 10µg pEGFP-N1 DNA or 10µg *gfp* mRNA or mock-nucleofected. Cells were either cultured without CD40L (immature) or with the immediate addition of CD40L. Day 5 immature human monocyte-derived DC were nucleofected with 10µg pEGFP-N1 DNA (B) or 10µg *gfp* mRNA (C) (filled histogram) or mock-nucleofected (empty histogram). Cells were either cultured without CD40L (immature) or with the immediate addition of CD40L. 24h post-nucleofection, cells were stained with PE-labeled mAB and analyzed by flow cytometry.

3.5.4 mRNA- but not DNA-nucleofected DC stimulate robust virus-specific T cell responses

A key issue in DC transfection for immunotherapy applications is the capacity to stimulate T cell responses to the specific transgene being introduced. This has been evaluated in the past using lipofection and electroporation approaches to deliver DNA or mRNA into monocyte-derived DC (248, 270); however, the ability to stimulate T cell responses following DNA and mRNA transfection of DC *via* nucleofection, which we have shown is a superior method of transfection, has not been compared. To evaluate this, we cultured monocyte-derived DC from a rhesus macaque that had a robust CTL response to simian immunodeficiency virus (SIV) Gag through vaccination (169). Immature DC were nucleofected with either pGag-N1 DNA encoding native SIVmac239 *gag* or *gag* mRNA (169) and immediately matured with CD40L for various intervals to induce maturation. Transfected DC were then cultured with autologous PBMC at a 1:10 ratio in a short-term IFN-γ ELISPOT assay to determine their capacity to stimulate Gag-specific effector T cells (169). DC that had been nucleofected with wild type *gag* mRNA 24 h earlier induced robust Gag-specific T cell responses with a frequency of greater than 5,000 IFN-γ spot-forming cells/million PBMC (Figure 5), similar to the frequency detected using DC pulsed with a pool of SIV Gag peptides (169). Importantly, DC that had been nucleofected with *gag*-expressing mRNA 48 h previously induced reduced but still strong T cell responses, supporting the notion that Ag expressed following mRNA delivery of transgene is processed and presented in DC for extended periods. In contrast to mRNA, DC nucleofected with DNA encoding wild type *gag* generated no detectable responses above the non-specific responses generated by DC nucleofected with p-EGFP-N1 DNA at either 24 h or 48 h post nucleofection (Figure 5). These data indicate that DC nucleofected with viral mRNA present Ag to T cells and stimulate robust

virus-specific T cell responses, whereas DC nucleofected with a comparable DNA construct are ineffective.

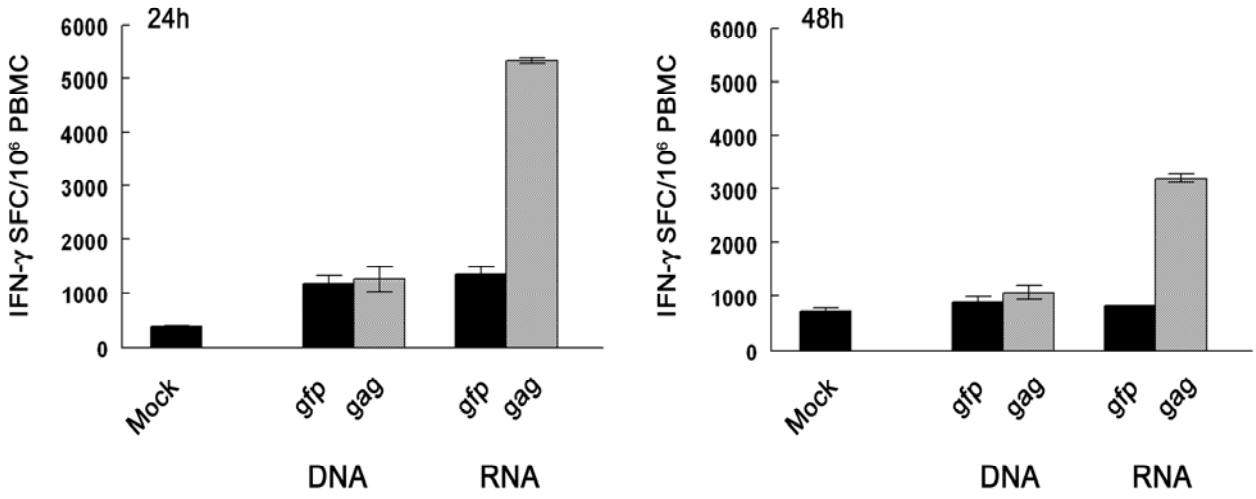

Figure 5. Monkey monocyte-derived DC nucleofected with wild-type SIV *gag* mRNA but not plasmid DNA encoding Gag stimulate Ag-specific T cells.Immature DC propagated from an SIV Gag-vaccinated monkey were mock-nucleofected or nucleofected with DNA or mRNA encoding GFP or SIVmac239 Gag as indicated and matured with CD40L for 24 h (left) or 48 h (right) prior to incubation with autologous PBMC in an IFN-γ ELISPOT assay. IFNγ spot-forming cells (SFC) were measured 24 h later. Shown are mean ± SEM of triplicate determinations.

3.6 DISCUSSION

Nonviral-based approaches for delivering genes to DC for cancer immunotherapy have distinct advantages over recombinant virus-based strategies, in particular because DNA or RNA can be amplified directly from tumor and serve as a source of polyvalent patient-specific Ag (12, 104, 249). This strategy is being employed in the design of therapeutic DC-based vaccines for HIV-1-infected individuals as well, as viral mRNA expressing patient-derived sequences or sequences from SIV-infected monkeys can readily be introduced into monocyte-derived DC for stimulation of autologous T cells (169, 266). Our studies confirm that nucleofection is superior to conventional electroporation and lipofection for introducing DNA and mRNA into human and monkey monocyte-derived DC, although nucleofection offered no advantage over electroporation when mRNA was introduced into the K562 cell line. However, mRNA was a significantly better source of Ag than was DNA for nucleofection of DC, producing higher level, more uniform and more durable Ag expression with limited toxicity.

The efficiency of nucleofection was such that as little as 5 ug mRNA resulted in almost complete transfection of DC, yet up to 20 ug mRNA could be safely delivered without compromising cell viability (data not shown). This provides the advantage of being able to introduce relatively small amounts of mRNA encoding multiple genes into DC simultaneously, such as different patient-derived viral genes for immunotherapy of HIV-1 infection. Alternatively, mRNA encoding individual virus or tumor Ag could be introduced together with genes designed to enhance DC function and Th1-stimulating capacity, such as OX40 ligand (59) or IL-12p70 (126), or to prolong DC survival, such as the gene encoding the anti-apoptotic protein Bcl-xl (111). In contrast, increasing the amount of DNA delivered by any means had a negative impact on DC viability, and introduction of only single gene into DC would be feasible using this approach.

Prior or concurrent maturation of human monocyte-derived DC with CD40L did not alter nucleofection efficiency with either DNA or mRNA in our studies, similar to previous reports (146, 178). It is possible that human and murine DC behave differently in this regard, as Larregina et al. reported that simultaneous transfection and CD40-mediated maturation of murine bone marrow-derived DC significantly enhanced DNA transfection efficiency (142). In fact, human DC nucleofected with DNA are refractory to maturation with CD40L, as we now show, or lipopolysaccharide (146). These effects are likely to be associated with the toxicity of DNA in DC, which results in reduction in the proportion of viable DC expressing Ag over time due to cell death (146). In contrast, DC nucleofected with mRNA were fully responsive to CD40 ligation, similar to other reports (146, 169). These studies suggest that mRNA-based delivery of genes will retain DC viability and responsiveness to maturation stimuli that are critical for T-cell stimulatory capacity, whereas delivery of Ag *via* DNA could potentially be deleterious.

A critical factor in gene transfection of DC for immunotherapy is the capacity for sustained presentation of Ag to tumor- or virus-specific T cells. We found that DC nucleofected with wild type SIV Gag Ag *via* mRNA nucleofection 24 h earlier stimulated strong Gag-specific effector T cell responses in a vaccinated monkey. Importantly, significant T cell responses were induced 48 h after introduction of mRNA into DC, reflecting the stable presentation of Ag in nucleofected cells. Similarly, B lymphoblastoid cells transfected with mRNA encoding a codon-optimized HIV-1 *nef* protein had undiminished capacity to stimulate Nef-specific T cells for at least 72 h post transfection (129). This durable Ag presentation by mRNA-transfected DC should be sufficient for DC to traffic to lymph nodes and engage Ag-specific T cells following intradermal or subcutaneous delivery to patients (177, 256). In contrast, DC nucleofected with DNA encoding Gag Ag were unable to induce a detectable Gag-specific T cell response in the same animal at

either 24 h or 48 h post transfection. Similarly, human DC expressing mRNA-encoded influenza matrix protein by electroporation were far superior in their capacity to stimulate M1-specific CTL as compared to DC expressing DNA-encoded Ag (248). In other studies, human DC transfected with DNA encoding human tumor-associated Ag were capable of stimulating Ag-specific CTL lines or priming autologous tumor-specific T cells (12, 262). It is possible in our studies that T cell stimulation would have been enhanced if DNA encoding codon-optimized viral Ag was used to transfect DC. However, we believe the use of wild type Ag derived directly from an infected individual provides a more relevant and robust test of DC-stimulatory capacity than does codon-optimized Ag, given the likely emphasis on autologous viral Ag in immunotherapy for HIV-1 (169, 266).

These data indicate that nucleofection of primary DC cultures with mRNA is an effective and non-perturbing means of delivering Ag for DC-based immunotherapy of cancer or infectious diseases, providing uniform and sustained Ag expression and presentation for stimulation of Ag-specific T cell. In contrast, while nucleofection is a more effective means of introducing DNA into DC than other methods, the resulting low viability, refractoriness to maturation, and poor T cell-stimulating capacity of transfected DC makes this approach highly unfavorable.

Acknowledgements. This work was supported by NIH grant AI52052 to S.M. Barratt-Boyes. We thank Nicole Banichar for assistance with animal procedures, and M. Murphey-Corb, A. Gambotto, P. Kalinski and C. Rinaldo for helpful suggestions.

4.0 CHAPTER TWO. ROBUST CD4$^+$ AND CD8$^+$ T CELL RESPONSES TO SIV USING MRNA-TRANSFECTED DC EXPRESSING AUTOLOGOUS VIRAL AG

Nada M. Melhem[1,2], Xiang Dong Liu[1,2], David Boczkowski[3], Eli Gilboa[3*], and Simon M. Barratt-Boyes [1,2,4]

[1] Center for Vaccine Research, University of Pittsburgh, Pittsburgh, Pennsylvania, 15261, USA

[2] Department of Infectious Diseases and Microbiology, Graduate School of Public Health, University of Pittsburgh, Pittsburgh, Pennsylvania, 15261, USA

[3] Department of Surgery, Duke University Medical Center, Durham, North Carolina, 27710, USA

[4] Department of Immunology, School of Medicine, University of Pittsburgh, Pittsburgh, Pennsylvania, 15261, USA

* Current address: Department of Microbiology and Immunology, School of Medicine, University of Miami, Miami, Florida, 33136, USA

Keywords: Dendritic cells, SIV, mRNA, T-cell stimulation

Correspondence: Dr. Simon M. Barratt-Boyes, Center for Vaccine Research, University of Pittsburgh, Pittsburgh, Pennsylvania 15261, USA. Phone: (412) 383-7537. Fax: (412) 624-4577. E-mail: smbb@pitt.edu.

Abbreviations: Lysosome associated membrane protein-1: LAMP-1; SFC: spot-forming cells

4.1 PREFACE

This chapter is adapted from a published manuscript in *Eur. J. Immunol*. 2007. 37: 2164-2173. Nada M. Melhem performed all the experimental. The manuscript was written by Nada M. Melhem. The generation of plasmids was done by Xiang Dong Liu. David Boczkowski and Eli Gilboa provided us with the pSP73 and pSP73/LAMP-1 constructs and gave us helpful suggestions. Simon M. Barratt-Boyes provided guidance and financial support.

Part of this study was presented as a poster at the AIDS Vaccine 2005 Conference, Montreal, Canada, September 2005. A short talk was presented at the Keystone Symposia 2006, Keystone, Colorado, March 2006 including another part of this work. Recently, this work was accepted as a poster at the AIDS Vaccine 2007 Conference, Seattle, Washington August 2007.

This chapter includes work in partial fulfillment of specific aim 2.

Eur. J. Immunol. 2007. 37: 2164-2173 :
http://dx.doi.org/10.1002/eji.200636782

4.2 SUMMARY

A potentially powerful strategy for therapeutic HIV-1 vaccination is the use of DC transfected with mRNA encoding autologous viral Ag, as epitopes presented by transfected DC would exactly reflect those expressed by infected cells in the individual. Using human and rhesus macaque monocyte-derived DC we show that nucleofection is a superior method for mRNA transfection resulting in high level protein expression and DC maturation. DC transfected with SIV *gag* isolated from an infected monkey stimulated robust Ag-specific recall T-cell responses of similar magnitude to those induced by peptide-pulsed PBMC that were predominantly $CD8^+$ T-cell mediated. Enhanced $CD4^+$ T -cell responses could be stimulated when Gag was redirected into the lysosomal pathway *via* the targeting signal derived from lysosome-associated membrane protein-1 (LAMP-1). Rhesus DC transfected with lysosome-targeted *gag* encoding an escape mutation in an immunodominant CTL epitope stimulated $CD4^+$ and $CD8^+$ T-cell responses of almost equivalent magnitude directed towards undefined epitopes outside of the mutated region. Finally, *gag*-transfected DC from SIV-infected monkeys stimulated significant Ag-specific recall T cell responses in an entirely autologous system. These findings demonstrate that mRNA-transfected DC expressing SIV Ag derived from infected monkeys stimulate broad and relevant T-cell responses, supporting this approach for therapeutic HIV-1 vaccine development.

4.3 INTRODUCTION

It is well appreciated that strong $CD4^+$ and $CD8^+$ T-cell immunity is required for control of HIV-1 infection (31, 136, 153, 223, 235) , and therapeutic vaccines designed to promote broad cellular immune responses are being actively pursued (275). However, an important challenge in the development of vaccines for HIV-1 is the diversity of clinical isolates (83) coupled with the propensity for the virus to undergo escape mutations in T-cell epitopes through selective pressure *in vivo* (92). Hence, CTL induced in response to the infecting strain may be incapable of controlling circulating virus as infection progresses (4, 32, 89, 198). To account for this complexity, a therapeutic vaccine should be tailored towards the autologous virus present in an individual at the time of immunotherapy.

DC are professional APC capable of Ag uptake, processing and presentation and are attractive candidates in the development of vaccines (13, 14). *In vitro* studies have demonstrated that DC transduced *via* viral vectors or pulsed with inactivated viral particles are effective at stimulating HIV-1-specific CTL responses (71, 156). Moreover, vaccination of rhesus macaques with DC expressing SIV or HIV-1 Ag induces broad virus-specific cellular responses which promote protection against pathogenic virus challenge (35, 155, 158, 190, 273). These prophylactic vaccine approaches have been based on well characterized strains of virus that may not be suitable for therapeutic vaccination as epitopes expressed by DC may not represent those in the infected individual. Efforts to include autologous viral Ag in therapeutic vaccines have relied on inactivated virus particles propagated from individual patients (157); however, this procedure is labor-intensive and may not uniformly lead to sufficient virus recovery for DC loading in all patients.

An attractive alternative source of viral Ag for therapeutic DC-based vaccination is mRNA, an approach that has been developed in the cancer immunotherapy field (139, 249). DC transfected with codon-optimized HIV-1 mRNA have been shown to induce strong T-cell immune responses *in vitro* (266, 279) with enhanced $CD4^+$ T-cell responses arising from DC expressing lysosome-targeted Ag (129). DC transfected with autologous viral mRNA isolated from HIV-1-infected individuals induce significant virus-specific T-cell responses (266). The rhesus macaque SIV model provides an ideal preclinical setting to test the therapeutic potential of DC-based vaccines using virus-derived mRNA. Here, we evaluated the capacity of mRNA-transfected DC to stimulate T cell responses against SIV using an *in vitro* system. We adopted a new electroporation method to generate high level expression of *gag* mRNA isolated during infection, and assessed the efficacy of mRNA-transfected DC bearing the lysosomal- associated membrane protein-1 (LAMP-1) targeting signal to induce both Ag-specific $CD8^+$ and $CD4^+$ T-cell responses. To provide a realistic test of the therapeutic potential of viral mRNA as an Ag source, we measured T-cell immunity induced by mRNA-transfected DC expressing a virus escape mutation acquired during infection.

4.4 MATERIALS AND METHODS

4.4.1 Plasmid and mRNA generation

gfp was subcloned from pGEM4Z/GFP/A64 (27, 28) into pSP73-Sph/A64 (183, 184) using BamHI to generate pSP73/GFP/A64. Similarly, *gfp* was subcloned into pSP73/gp96ss/LAMP-1/A64 using Sfil to generate pSP73/GFP-LAMP-1/A64. For the initial generation of Gag-encoding plasmids, PBMC were isolated from a rhesus macaque 2-weeks post infection with SIVmac239 and cellular DNA extracted using DNeasy tissue kit (Qiagen, Chatsworth, CA). For all other Gag-expressing plasmids, cell-free plasma was isolated from monkeys infected with SIVmac251 or SIV/DeltaB670 and viral RNA isolated using QIAMP viral RNA kit (Qiagen). Samples from a total of 12 infected monkeys with virus loads ranging from 10^2 to 5.5 x 10^6 RNA copies/ml plasma were used over the course of the study. Approval was obtained from the institutional animal use and care committee for all experiments involving rhesus macaque samples. The *gag* gene from each virus was amplified by PCR using gene-specific primers and subsequently subcloned into the pSP73 vector to generate pSP73/mac239Gag/A64, pSP73/mac251Gag/A64, pSP73/B670Gag(T182I)/A64 and pSP73/B670Gag/A64, depending on the particular virus isolate. Influenza HA was tagged to SIVmac239 Gag to generate pSP73/Gag-HA/A64. *gag* from SIVmac239, SIV/DeltaB670 inoculum and SIV/DeltaB670 Gag (T182I) were additionally cloned into the pSP73-LAMP-1 vector to generate pSP73/GAG-LAMP-1/A64, pSP73/B670GAG-LAMP-1/A64 and pSP73/B670GAG (T182I)-LAMP-1/A64, respectively. *gag* sequences encoding amino acids 159-252 from SIV/DeltaB670 encompassing either wild-type or mutated CM9 sequences were subcloned into pSP73 as above to generate pSP73/B670Gag159-252/A64 and pSP73/B670Gag159-252(T182I)/A64, respectively. For generation of mRNA,

pSP73/GFP/A64 was linearized with SpeI, whereas all Gag-expressing plasmids were linearized using NotI. *In vitro* transcription was done with T7 polymerase using the mMessage Machine kit (Ambion, Austin, TX). Purification of *in vitro* transcribed mRNA was performed by DNase I digestion followed by LiCl precipitation and a 70% ethanol wash. The quality of mRNA was checked by agarose formaldehyde gel electrophoresis. mRNA concentration was assayed by spectrophotometry (GeneQuant pro, Amersham Pharmacia Biotech, Little Chalfont, UK) at OD_{260}.

4.4.2 Propagation and transfection of DC

Approval was obtained from the institutional review board prior to experiments involving human samples. DC were cultured from purified blood monocytes of SIV-naïve or SIV-infected rhesus macaques or healthy human volunteers using 1,000 U/ml GM-CSF (Berlex Laboratories, Inc., Richmond, CA) and 1,000 U/ml IL-4 (Schering-Plough, Kenilworth, NJ) as described (22). DC were transfected at day 5 of culture. For transfection using the TransFast^{TM} Transfection reagent (Promega, Madison, WI) or TransMessenger reagent (Qiagen, Valencia, CA), 4 µg GFP mRNA was used with Opti-MEM (Gibco Invitrogen Corporation, Frederick, MD) and other reagents provided by the manufacturers and 10^6 DC transfected as per each manufacturer's instructions. For electroporation, 2 x 10^6 DC in Opti-MEM were mixed with 10 µg GFP mRNA and electroporated in a 0.4-cm cuvette using the Gene Pulser II Electroporation System (Bio-Rad, Hercules, CA) at a voltage of 300 V and a capacitance of 150µF. For nucleofection using Amaxa nucleofector (Amaxa, Koln, Germany), 10-20 µg mRNA was added to 1-2 x 10^6 DC in Opti-MEM and transfected in a 2 mm-wide electroporation cuvette (BTX, San Diego, CA) using the U-02 program. Smaller amounts of mRNA were used for lipofection as compared to

electroporation and nucleofection as toxicity was noted at higher amounts with the former methods (data not shown). Following transfection with the various methods, cells were cultured in fresh RPMI with 10% FCS, L-glutamine, sodium pyruvate, nonessential amino acids, penicillin-streptomycin and 10 mM HEPES buffer (complete media) supplemented with GM-CSF and IL-4 for 24 h. In some experiments DC were matured for 24 h immediately following transfection with 3 μg/ml recombinant trimeric CD40L (Immunex, Seattle, WA) as described (22).

4.4.3 Flow cytometric analysis

GFP expression was measured in transfected DC by flow cytometry at various intervals after transfection using a Beckman Coulter cytometer (Miami, FL). For immunophenotyping of DC, cross-reactive mAb to human HLA-DR, CD80, CD83, CD86 (all from BD Pharmingen, San Diego, CA) and CD40 (Ancell, Bayport, MN) were used as described (22).

4.4.4 Detection of Gag expression

24 h post-transfection with *gag* or *gag-HA* mRNA, monkey DC were lysed and lysates were separated by gel electrophoresis and transferred to nitrocellulose membrane or polyvinylidine difluoride membrane (Bio-Rad), respectively. Gag p17 was detected by probing cell lysates with p17-specific mAb KK59 followed by HRP-conjuguated anti-mouse IgG (Bio-Rad). Gag-HA was detected by mAb anti-HA-Peroxidase (12CA5) (Roche Applied Science, Indianapolis, IN). Development was performed using the Immun-Star^{TM} HRP Substrate kit (Bio-Rad).

4.4.5 ELISPOT assay

Previously frozen PBMC were pulsed with pools of 15-mer peptides spanning the entire sequence of SIVmac239 Gag protein or influenza HA (Sigma Genosys, Woodlands, TX) and IFN-γ production detected 24 h later by ELISPOT assay as described (21). For assays using peptide-pulsed DC, monkey DC were matured with CD40L for 24 h as above and incubated with Gag or HA peptides (5 μg /ml) for 1 h at 37^{o}C. Cells were washed and incubated with PBMC at a 1:10 ratio in the ELISPOT assay. For experiments using mRNA transfected DC, DC were transfected with *gag* or *gfp* mRNA or mock transfected (nucleofected in the absence of mRNA) and matured with CD40L for 24 h prior to incubation with PBMC. Where indicated, transfected DC were incubated with PBMC depleted of $CD4^+$ T cells (CD4 microbeads, Miltenyi Biotec, Auburn, CA) or $CD8^+$ T cells (CD8 microbeads, Miltenyi Biotec), as described (35). Depletions were >90% efficient for each T-cell subset (35). The same total number of cells was added to wells irrespective of depletion conditions.

4.4.6 Statistics

We used non-parametric tests (Wilcoxon) to test the difference in median between autologous Gag-specific T –cell responses and GFP-induced T-cell responses.

4.5 RESULTS

4.5.1 Human monocyte-derived DC express high levels of Ag following nucleofection

Given the variety of approaches that have been devised for DC transfection, from lipid- based to electroporation based-methods, we first sought to optimize mRNA transfection of DC. In initial experiments, we used human monocyte-derived DC transfected with *gfp* mRNA generated from the linearized plasmid pGEM4Z/GFP/A64 (27). DC were propagated from purified monocytes (22) and cultured for 5 days at which time they were transfected by standard electroporation, TransFast™ lipofection, TransMessenger lipofection and nucleofection. Expression of GFP was measured 24h later by flow cytometry. Transfection efficiency ranged from no to moderate expression of GFP following the standard methods of electroporation and lipofection consistent with earlier reports (248) (Figure 6A). In contrast, 92% of DC expressed GFP following nucleofection with mRNA, similar to findings in murine macrophages (265) (Figure 6A). GFP expression was detected as soon as 3h and peaked at 24h post-nucleofection (Figure 6B). 48h post-nucleofection, 70% of human DC still expressed GFP, similar to previous findings (269).

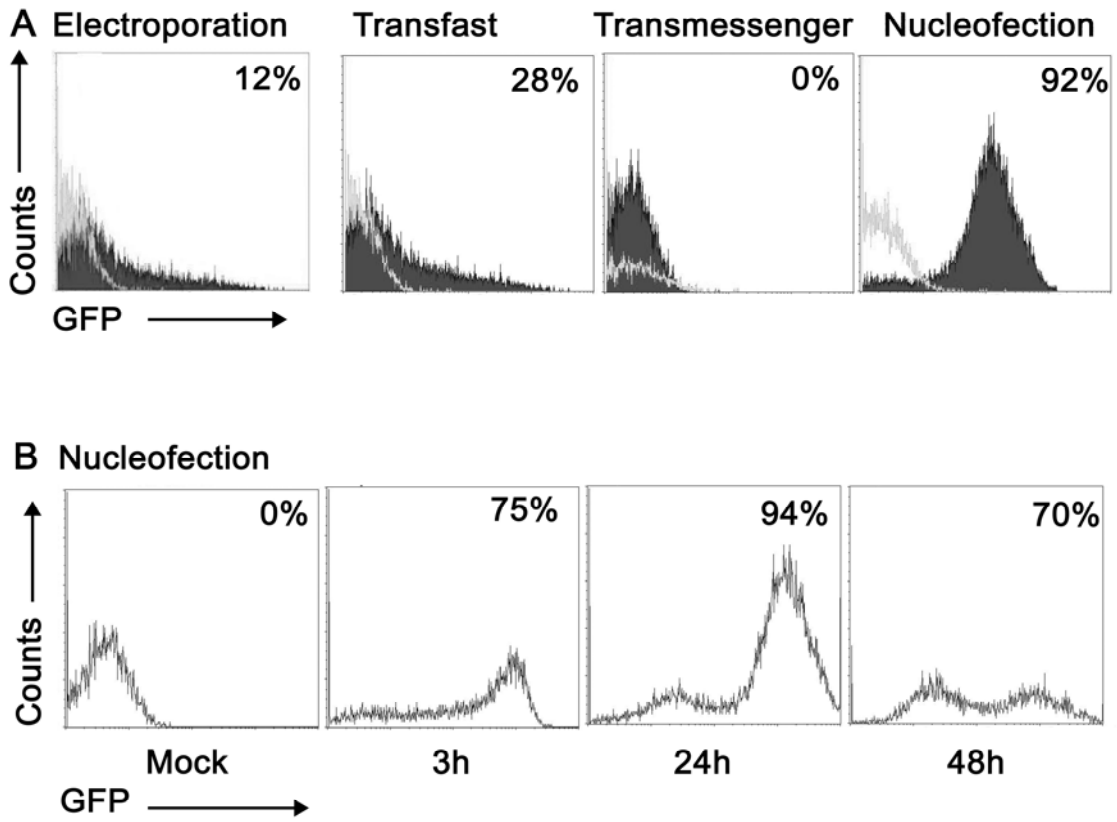

Figure 6. Efficient mRNA transfection of human monocyte-derived DC via nucleofection. (A) Immature human monocyte-derived DC were transfected with *gfp* mRNA (filled histogram) or mock transfected (empty histogram) using electroporation, Transfast™ lipofection, TransMessenger Transfection reagent or nucleofection, and GFP expression determined 24h later. (B) Immature human monocyte-derived DC were nucleofected with *gfp* mRNA or mock-nucleofected and expression of GFP determined at 3, 24 and 48h post-nucleofection. Numbers represent the percent of DC transfected based on mock transfection.

4.5.2 Efficient mRNA transfection of DC from SIV-infected monkeys

We next evaluated the capacity of nucleofection to transfect rhesus macaque monocyte-derived DC. As for human DC, DC cultured from blood of SIV-naïve monkeys were readily transfected with mRNA, with 73% of cells expressing GFP protein within 24 h (Figure 7A). Given that our goal is to use mRNA-transfected DC during SIV infection, we next determined whether SIV infection itself affects the capacity for DC to be transfected with mRNA. DC were propagated from pooled cryopreserved monocytes taken between 3 and 45 weeks post infection of a rhesus macaque with SIV/DeltaB670 (virus load ranged from 10^2 to 5 x 10^5 RNA copies/ml plasma over this time), and from monocytes collected at 27 weeks post infection from a macaque with neuroAIDS following SIVmac251 infection (virus load 1.2 x 10^6 RNA copies/ml plasma), and nucleofected with *gfp* mRNA as above. There was no evidence for reduction in mRNA transfection in either sample, as evidenced by high level GFP expression (Figure 7A). These data indicate that SIV infection does not impact the capacity for monocyte-derived DC to be transfected, even when cells are propagated from animals in the advanced stages of disease. We next evaluated the effect of diverting Ag into the lysosomal/endosomal pathway on protein expression in transfected DC. We first subcloned *gfp* from pGEM4Z/GFP/A64 into the pSP73/gp96ss/LAMP-1/A64 vector encoding the targeting signal of LAMP-1. SIV-naïve rhesus macaque monocyte-derived DC were then nucleofected with mRNA expressing GFP with and without LAMP-1 targeting signal and analyzed at 24h post-nucleofection. GFP expression was substantially decreased in DC transfected with endosome-targeted GFP as compared to controls, dropping from 88% to 15% (Figure 7B). These data are consistent with a rapid processing of endogenously produced protein when it is directed into the endosome (226).

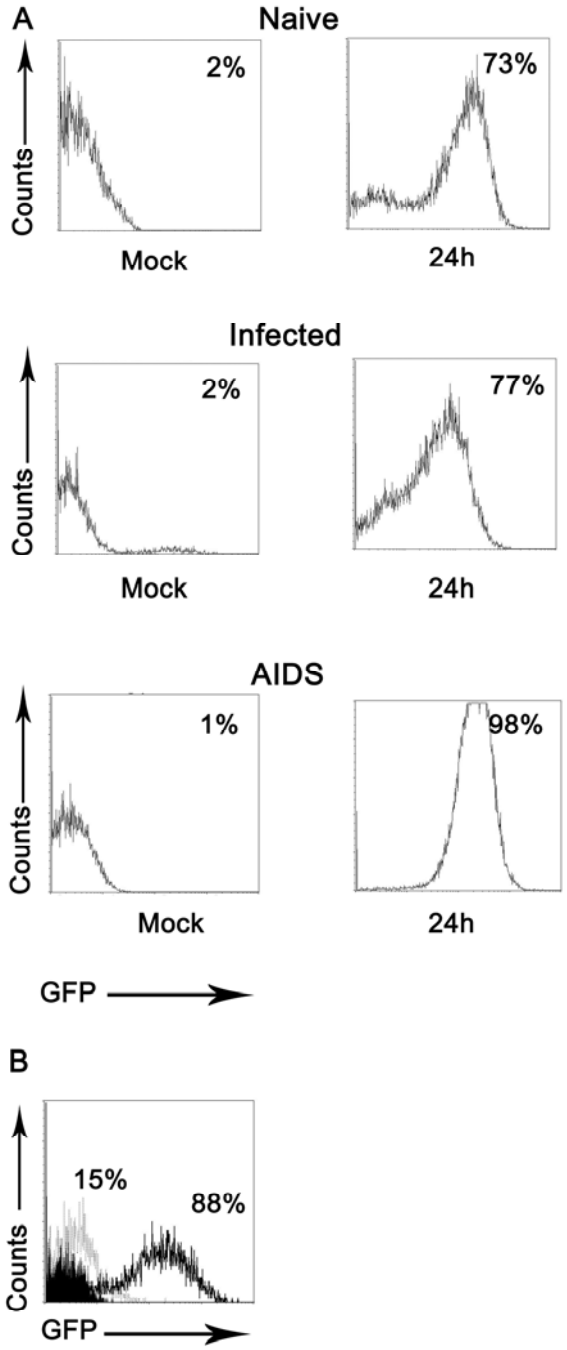

Figure 7. Efficient mRNA transfection of DC propagated from monkeys with and without SIV infection.
(A) DC were propagated from an SIV-naïve macaque (top), from pooled monocytes taken from a macaque at 3 – 45 weeks post SIV infection (middle) or from monocytes taken 27 weeks post SIV infection from a monkey with neuroAIDS (bottom), and nucleofected with *gfp* mRNA or mock-nucleofected. Expression of GFP was determined 24 h later. **(B)** Monocyte-derived DC propagated from an SIV-naïve rhesus macaque were nucleofected with *gfp* mRNA (bold line), *gfp-LAMP-1* mRNA (dotted line) or mock-nucleofected (filled histogram) and expression of GFP determined 24h later. Numbers represent the percent of DC nucleofected based on mock-transfection.

4.5.3 mRNA nucleofection induces phenotypic DC maturation

Transfection of immature human DC with mRNA *via* lipofection but not electroporation has been reported to induce maturation (43, 103, 128, 269, 278). To test the effect of mRNA nucleofection on maturation of DC, we analyzed the phenotype of nucleofected monkey DC with and without stimulation with CD40L. Nucleofection of immature DC with *gfp* mRNA, but not mock nucleofection, led to expression of CD80 and CD83 and upregulation of CD86 and CD40 indicative of maturation (Figure 8). Expression of CD83 was moderately enhanced when mock- or mRNA–transfected DC were exposed to CD40L based on a more pronounced shift in CD83 expression (Figure 8). These data indicate that mRNA transfection induces DC maturation, but that other exogenous stimuli known to induce maturation may provide additional benefits.

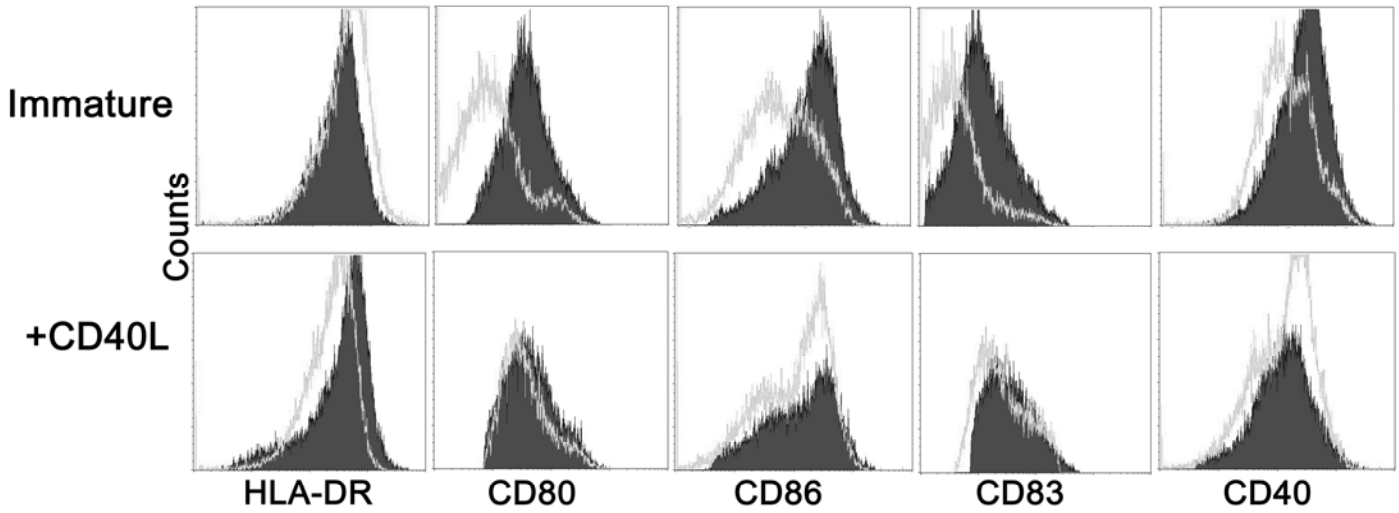

Figure 8. Nucleofection of monkey monocyte-derived DC results in phenotypic maturation. Day 5 immature monocyte-derived DC propagated from an SIV-naïve rhesus macaque were nucleofected with *gfp* mRNA (filled histogram) or mock-nucleofected (empty histogram), and then cultured without CD40L (upper row) or with the immediate addition of 3 µg/ml CD40L (lower row). At 24h post-nucleofection, cells were stained with PE-labeled mAb as shown and analyzed by flow cytometry.

4.5.4 Efficient T cell stimulation using DC expressing wild-type *gag* mRNA

We next sought to determine whether wild-type, non codon-optimized SIV *gag* mRNA could be efficiently expressed in monkey DC, which is an important criterion for using autologous virus as a source of Ag. To test for expression of Gag protein in mRNA-transfected DC, we isolated PBMC from a rhesus macaque 2 weeks post-infection with SIVmac239 and amplified *gag* from cellular DNA. Virus replication *in vivo* over this 2-week period had no impact on the sequence of the *gag* gene, as no mutations were detected when compared to the published SIVmac239 Gag sequence (data not shown). *gag* cDNA was subsequently cloned into pSP73-Sph/A64 and mRNA generated for nucleofection of immature DC. 24h post-nucleofection, Gag protein was readily detected in cell lysates as determined by western blotting with SIV p17-specific mAb KK59 (Figure 9A). Similarly, Gag protein could be detected in lysates following transfection of cells with *gag* mRNA linked to an influenza hemagglutinin (HA) tag and labeling with an HA-specific mAb (Figure 9A).

To determine the capacity of mRNA-transfected monkey DC to stimulate Gag-specific T-cell responses, we nucleofected SIVmac239 *gag* mRNA into DC propagated from a healthy monkey (R187) that had been immunized with a recombinant adenovirus-based vaccine (21). This monkey expressed the MHC class I allele Mamu-A*01 and had a robust CTL response to the immunodominant SIV Gag epitope $CM9_{181\text{-}189}$ (173) (data not shown). SIV *gag* mRNA-transfected DC stimulated a strong IFN-γ response from autologous PBMC that was Gag-specific; as negligible T cell reactivity was noted when PBMC were stimulated either with *gfp* mRNA-transfected or mock-transfected DC (Figure 9B). This level of T-cell reactivity was similar to that induced when PBMC were pulsed with peptides spanning the entire SIVmac239 Gag protein, the standard method for inducing virus-specific T-cell responses (21) (Figure 9B). DC pulsed with

Gag peptides, but not a control panel of HA peptides, generated slightly more robust responses (Figure 9B). We next addressed whether these recall responses were $CD4^+$ or $CD8^+$ T-cell mediated and the effect of redirecting Gag to the lysosome on these responses. DC from R187 nucleofected with SIVmac239 *gag* mRNA induced Gag-specific T-cell responses that were almost exclusively $CD8^+$ T-cell mediated based on cell depletion experiments (Figure 9C). In contrast, DC nucleofected with *gag-LAMP-1* mRNA produced similarly strong $CD8^+$ T-cell mediated responses but a greater $CD4^+$ T-cell responses to Gag (Figure 9C). The enhanced T-cell response noted with either *gag* or *gag-LAMP-1* transfection when $CD4^+$ T cells were depleted is likely due to the concurrent enrichment of $CD8^+$ T cells in these conditions, as found in our previous studies (21, 35). Together, these data demonstrate that DC nucleofected with wild-type SIV *gag* mRNA efficiently present Ag to virus-specific T cells and that redirecting Gag Ag to the lysosomal pathway enhances $CD4^+$ T-cell responses, consistent with previous reports (129, 251, 250).

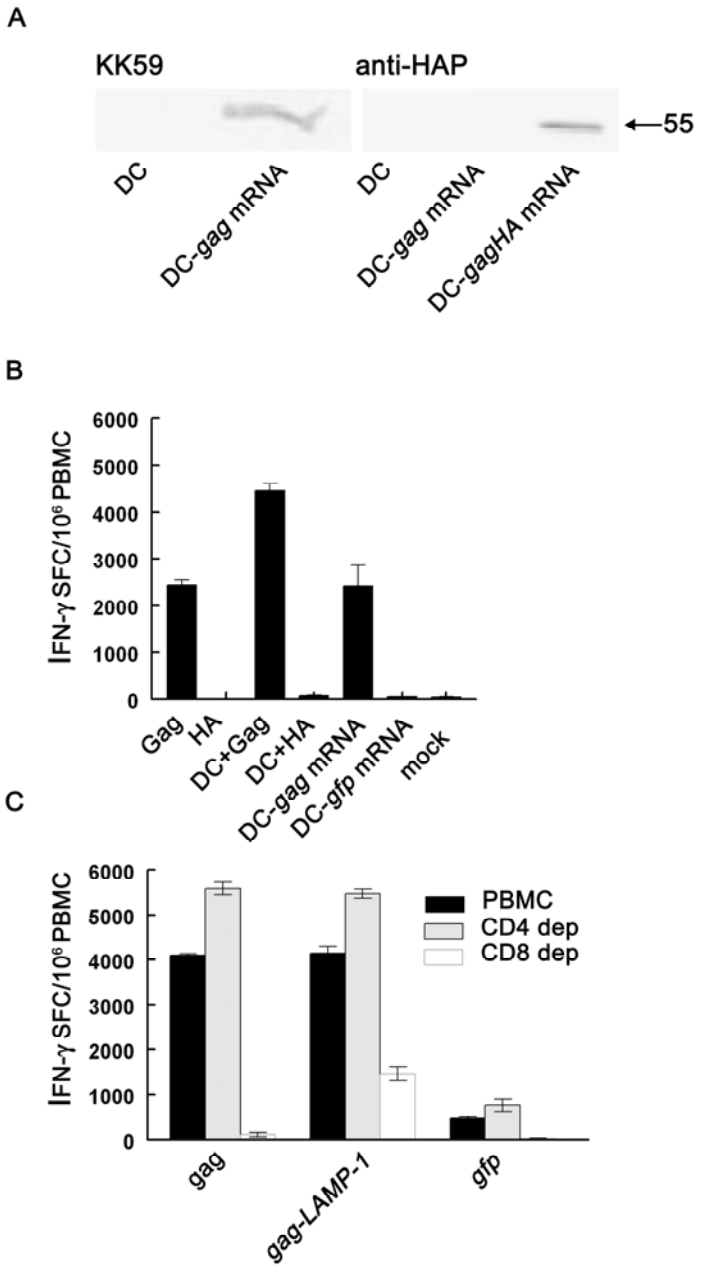

Figure 9. DC nucleofected with wild-type SIV *gag* mRNA stimulate antigen-specific T cells.
(A) Left panel: Immature monkey monocyte-derived DC were nucleofected with SIV *gag* mRNA isolated from an infected monkey or mock-nucleofected and cell lysates probed with p17-specific mAb KK59 24h later. Right panel: Immature monkey monocyte-derived DC were nucleofected with SIV *gag* mRNA, SIV *gag-HA* mRNA or mock-nucleofected and cell lysates probed with anti-HA mAb 24 hours later. **(B)** PBMC from a healthy Gag-vaccinated Mamu-A*01-expressing rhesus macaque (R187) were pulsed with pools of Gag or HA peptides, or incubated with DC pulsed with Gag or HA peptides, or DC nucleofected with SIV mac239 *gag* or *gfp* mRNA, and IFN-γ production detected 24h later by ELISPOT. Shown are mean ± SEM of triplicate determinations. **(C)** DC from R187 were nucleofected with mRNA encoding SIVmac239 *gag, gag-LAMP-1* or *gfp* and incubated with autologous PBMC with or without Ab-mediated depletion of $CD4^+$ or $CD8^+$ T cells in an ELISPOT assay. Shown are mean ± SEM of triplicate determinations. SFC=spot-forming, dep=depleted.

4.5.5 T cell stimulation using DC expressing Gag encoding a CTL escape mutation

The advantage of using autologous virus as a source of Ag in a therapeutic HIV-1 vaccine is the capacity to present relevant epitopes to the immune system, particularly in the event of virus escape. To evaluate this capacity in our system, we next expressed *gag* isolated from a Mamu-A*01-expressing macaque M2201 that contained a threonine to isoleucine escape mutation at amino acid 182 within the immunodominant CM9 epitope 15 weeks after infection with the uncloned pathogenic isolate SIV/DeltaB670 (21). To provide a relevant comparison to previous experiments, we continued to use DC and PBMC from the healthy Gag-vaccinated Mamu-A*01-expressing macaque R187. As expected, DC nucleofected with mRNA encoding SIVmac239 *gag* or *gag* cloned from SIV/DeltaB670 inoculum (B670 *gag*) stimulated robust Gag-specific recall T-cell responses (Figure 10A). In contrast, DC transfected with *gag* mRNA isolated from animal M2201 at 15 weeks post-infection and encoding the CM9 epitope escape mutation (referred to as B670 *gag (T182I)*), induced substantially reduced T-cell responses (Figure 10A). *gfp* mRNA-transfected or mock-transfected DC stimulated background IFN-γ release from PBMC, as expected (Figure 10A). To document that the T-cell response to DC transfected with B670 *gag (T182I)* mRNA was directed to epitopes other than CM9, we generated shorter sequences of mRNA from the inoculum and escape viruses. DC transfected with B670 *gag159-252* mRNA, encoding intact CM9 but no other known Mamu-A*01-restricted CTL epitopes, stimulated strong T-cell responses, whereas DC transfected with B670 *gag159-252(T182I)* mRNA encoding mutant CM9 induced no detectable response, similar to *gfp*- or mock-transfected DC (Figure 10A). Similar results were obtained when DC were pulsed with wild-type CTPYDINQM peptide as compared to peptide CIPYDINQM containing the threonine to isoleucine mutation at position 2 (data not shown), indicating that the difference in T cell

response was not due to differential expression of the various mRNA constructs. These findings confirm that DC transfected with *gag* mRNA containing an escape mutation in the immunodominant CM9 epitope presented intact subdominant epitopes for stimulation of T cells.

We next determined the impact of lysosome- targeting of Gag protein on the resulting T-cell responses to mutant Gag. DC from R187 nucleofected with B670 *gag* but not *gfp* induced Gag-specific T-cell responses that were primarily $CD8^+$ T-cell mediated, as expected. In contrast, both $CD8^+$ and $CD4^+$-mediated T-cell responses were induced by DC nucleofected with B670 *gag-LAMP-1* mRNA (Figure 10B). The effect of lysosome targeting on the $CD4^+$ T cell response was even greater when *gag* mRNA encoding the mutated CM9 epitope was expressed as a lysosomal protein *via* B670 *gag (T182I)-LAMP-1* mRNA, with almost equivalent $CD4^+$ and $CD8^+$ T-cell responses being generated (Figure 10B).

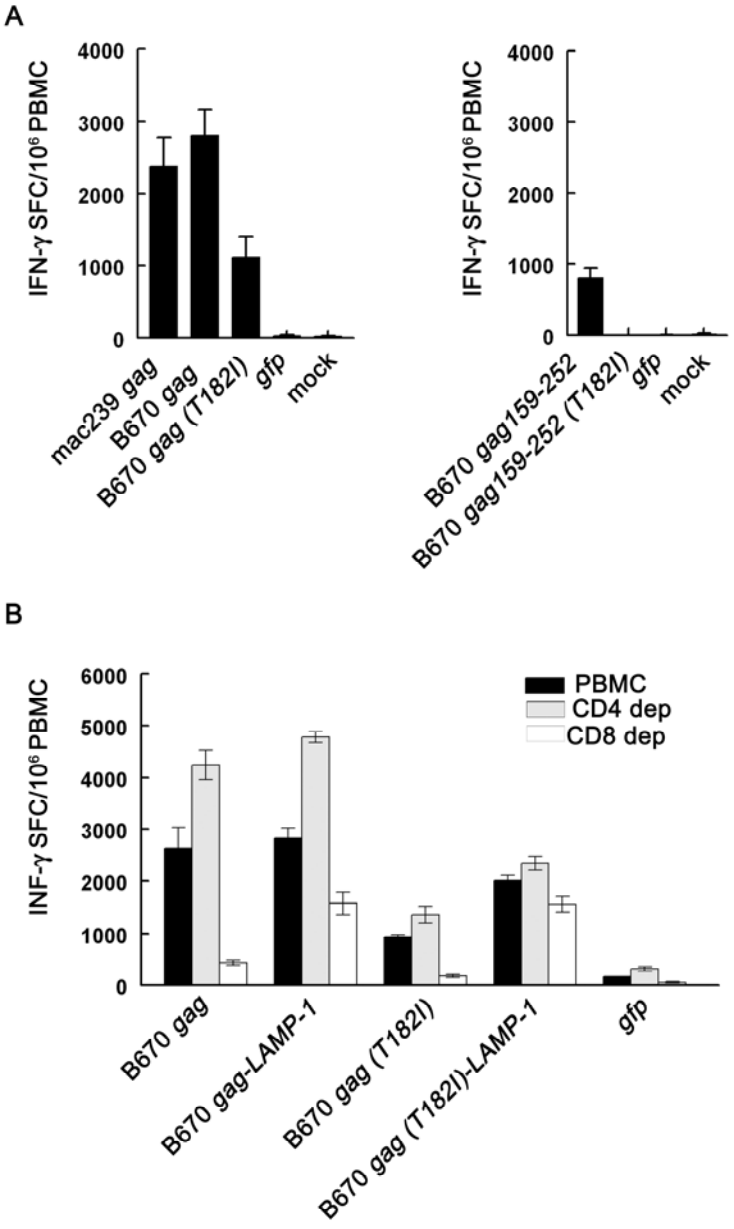

Figure 10. DC nucleofected with SIV *gag* or *gag-LAMP-1* mRNA encoding a mutated immunodominant CTL epitope induce T-cell responses to undefined subdominant epitopes mediated by both $CD4^+$ and $CD8^+$ T cells. **(A)** Left: DC from a healthy Gag-vaccinated Mamu-A*01-expressing rhesus macaque (R187) were nucleofected with mRNA encoding SIVmac239 *gag,* SIV/DeltaB670 *gag* (B670 *gag)*, SIV/DeltaB670 *gag* with T182I mutation (B670 g*ag* (T182I)), or *gfp* and used in an ELISPOT assay with autologous PBMC. IFN-γ producing cells were enumerated 24h later. Right: DC from R187 were nucleofected with mRNA encoding SIV/DeltaB670 gag159-252 *(*B670 *gag159-252)*, SIV/DeltaB670 gag159-252 with T182I mutation (B670 *gag159-252* (T182I)) or *gfp* and used in an ELISPOT assay as above. **(B)** DC from R187 were nucleofected with B670 *gag*, B670 *gag-LAMP-1*, B670 *gag (T182I)*, B670 *gag(T182I) –LAMP-1* or *gfp* mRNA and used with autologous PBMC with and without Ab-mediated depletion of $CD4^+$ or $CD8^+$ T cells in an ELISPOT assay as above. Shown are mean ± SEM of triplicate determinations. SFC=spot-forming, dep=depleted.

4.5.6 DC expressing autologous *gag* mRNA stimulate Ag-specific T-cell responses

We next tested the ability of mRNA-transfected DC to stimulate virus-specific T-cell responses in an entirely autologous system. We first determined the ease with which *gag* could be amplified from blood of SIV-infected monkeys. We were able to recover *gag* from cell-free plasma from 11/11 SIV-infected monkeys with virus loads as low as 10^2 RNA copies/ml plasma (data not shown), indicating that recovery of *gag* from infected plasma is remarkably efficient. DC were subsequently propagated from 3 macaques infected with SIV/DeltaB670 (M2201, M2301, and M7801) and 2 macaques infected with SIVmac251 (R484, R486). Plasma harvested at week 15 post-infection from the first group and week 4 post-infection from the second group was used to amplify the predominant *gag* sequence from viruses present in each animal. *gag* was then subcloned into the pSP73-Sph/A64 vector for mRNA generation and DC transfection. mRNA- or mock-transfected DC were then incubated with autologous PBMC from each animal in an ELISPOT assay. DC transfected with autologous *gag* mRNA induced greater T-cell responses than DC transfected with *gfp* mRNA in each animal (Figure 11A), and this difference was statistically significant when compared across all animals combined (P=0.043; Figure 11B). Notably, in animals M7801 and M2201 which provided sufficient DC for additional control conditions, *gfp*- and mock-transfected DC stimulated similar background levels of IFN-γ release from autologous PMBC (Figure 11A). This finding confirms that mRNA transfection in itself did not negatively or positively impact the capacity of DC to stimulate T cells. The magnitude of individual responses to Gag was not associated with virus load (data not shown). These data confirm that DC transfected with mRNA encoding Gag Ag isolated during infection can stimulate significant autologous T-cell responses.

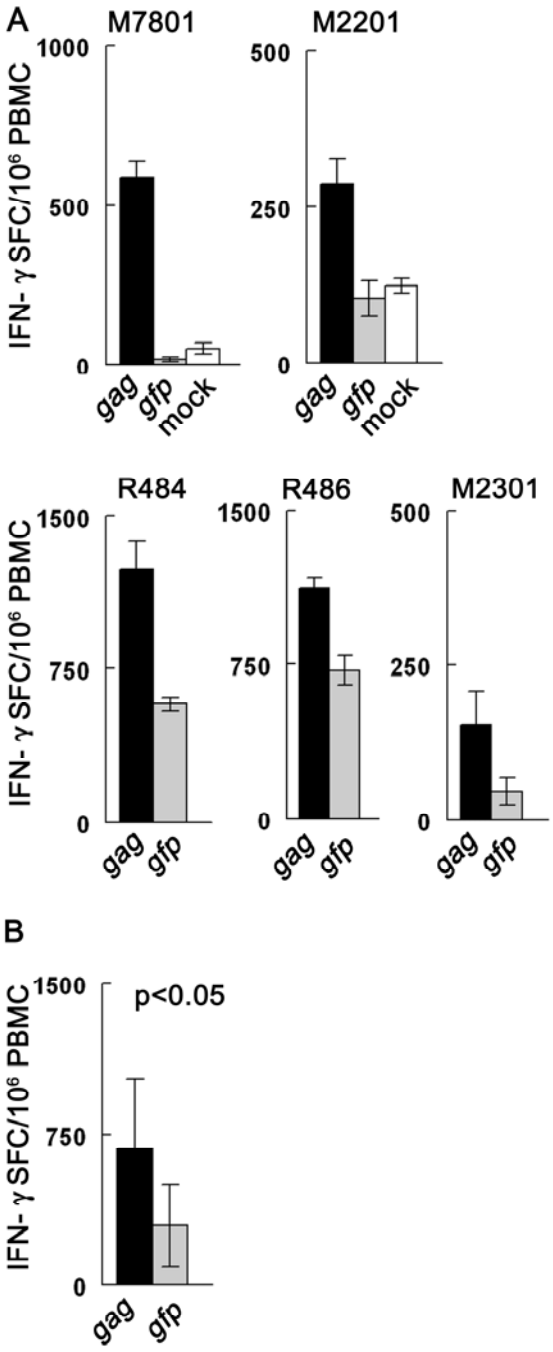

Figure 11. DC nucleofected with *gag* mRNA isolated from infected monkeys induce significant Gag-specific T-cell responses from autologous PBMC.

(A) Immature DC propagated from SIV-infected rhesus macaques as shown were nucleofected with mRNA encoding autologous *gag* or *gfp* (five animals) or mock-nucleofected (two animals) and used as stimulators of autologous PBMC in an IFN-γ ELISPOT assay. Shown are mean ± SEM of triplicate determinations. **(B)** Comparison of IFN-γ release from autologous PBMC in response to *gag*- or *gfp*-transfected DC for all five animals combined. Shown are mean ± SEM of triplicate determinations. SFC: spot-forming cells.

4.6 DISCUSSION

The need for a therapeutic vaccine for HIV-1 is apparent given the inability of antiretroviral therapy to eradicate virus in infected individuals (48, 75). However, virus diversity coupled with emergence of escape mutants during the course of infection in infected individuals suggest that the source of Ag in such vaccines is critical (2, 92, 274). Here we complement recent *in vitro* studies in humans (129, 266) by showing that monkey monocyte-derived DC expressing viral mRNA isolated during SIV infection stimulate strong and broad virus-specific T-cell responses even in the face of virus escape, with an enhancement of the $CD4^+$ T-cell response seen when Ag is targeted to the lysosome.

We found that nucleofection was a superior method to introduce mRNA into monkey and human monocyte-derived DC, with electroporation and lipofection providing relatively poor levels of Ag expression. Nucleofection has been used for DNA transfection of human DC and cytokine-induced killer cells (56, 182) as well as mRNA transfection into primary rat neurons and murine macrophages cell lines (82, 265). Other reports have indicated that electroporation is relatively efficient at transfecting human DC with mRNA although results have been somewhat variable (129, 229, 266, 269). mRNA transfection of immature monocyte-derived DC by nucleofection induced significant maturation as evidenced by expression of CD83 and CD80 and upregulation of CD40 and CD86. Similarly, DC transfected with mRNA *via* lipofection or simple pulsing undergo phenotypic maturation (43, 103, 128, 279). DC maturation is thought to be mediated by mRNA binding to endosomal TLR3 by regions of double stranded secondary structures (43, 128). In contrast, mRNA transfection of human monocyte-derived DC *via* electroporation does not appear to induce maturation (269) although the reason for this discrepancy is unclear. Despite the maturation induced through mRNA nucleofection we found a

modest enhancement of expression of CD83 following ligation of CD40, suggesting that maturation *via* mRNA transfection can be augmented by other stimuli. It is likely that agonists of other TLR such as TLR8 may synergize with mRNA to activate DC, as combined ligation of TLR3 and TLR8 appears to produce optimal maturation of myeloid DC (187).

We found that recovery of virus cDNA for mRNA generation is an efficient process even when samples are isolated from monkeys with very low virus loads, indicating that this approach may be preferable to cell culture techniques for the generation of autologous virus Ag (157). However, the use of autologous virus Ag in itself is technically demanding, requiring the recovery of virus sequences from each individual. Nevertheless, autologous virus Ag does offer several potential advantages for immunotherapy over alternatives based on artificial sequences encoding a centralized (consensus or ancestral) gene, which have been put forward as a vaccine strategy to address HIV-1 diversity (83). Firstly, using DC transfected with autologous virus Ag expressing mutated immunodominant CTL epitopes leads to stimulation of CTL specific for subdominant epitopes, as we have shown, and such epitopes are known to be important in virus control (76). While conserved subdominant epitopes would also be expressed by centralized sequences, using the latter in therapeutic vaccines in the face of immune escape could have a negative effect, as CTL specific for immunodominant epitopes present in the consensus sequence will be preferentially expanded (204). This may occur even as long as 4 weeks following complete replacement of circulating virus with escape mutants (21). Secondly, de-novo generation of novel CTL epitopes can arise from mutated immunodominant epitopes (6), and these novel epitopes would not be presented by DC transfected with centralized sequences. The value of reagents based on autologous virus over consensus sequences in stimulating enhanced CTL responses to variable regions of HIV-1 has been demonstrated *in vitro* using comprehensive

panels of overlapping peptides (7). Whether autologous virus Ag provides a clinical advantage over centralized Ag awaits comparative DC-based immunotherapy studies in HIV-1-infected individuals.

It is important in therapeutic vaccination for HIV-1 that virus-specific $CD4^+$ T-cell responses be expanded, as these are critical in maintenance of CTL (205, 223, 227). Our results indicate that lysosome-targeting of protein leads to reduced expression of the intact protein in transfected cells, but enhanced stimulation of virus-specific $CD4^+$ T cells, consistent with previous findings (129). The effect of targeting viral Ag to the lysosome on the relative magnitude of the $CD4^+$ T-cell responses was amplified with the loss of an immunodominant CTL epitope naturally acquired during SIV infection. Overall, these data support the use of lysosome-targeted autologous viral Ag in DC-based therapeutic vaccination for HIV-1.

Acknowledgements. This work was supported by National Institutes of Health grant AI52052 to S.M. Barratt-Boyes. The authors thank D. Slovitz for technical assistance, A. Trichel and D. McClemens-McBride for assistance with animal procedures, T. Kodama for SIVmac239-infected blood samples, and D.J. Perkins, P. Gupta and C. Rinaldo for access to equipment. The KK59 mAb and peptides representing SIVmac239 Gag protein were provided by the National Institutes of Health AIDS Research and Reference Reagent Program.

5.0 CHAPTER THREE. IMPACT OF COSTIMULATORY TUMOR NECROSIS FACTOR RECEPTOR FAMILY MOLECULES OX40L AND CD70 ON THE IMMUNOSTIMULATORY CAPACITY OF MONKEY MONOCYTE-DERIVED DC

Nada M. Melhem,[1,2] Xiang Dong Liu,[1,2] and Simon M. Barratt-Boyes[1,2,3]

[1] Center for Vaccine Research and [2]Departments of Infectious Diseases and Microbiology and [3]Immunology, University of Pittsburgh, Pittsburgh, Pennsylvania, 15261, USA

5.1 PREFACE

The following work was performed to elucidate the effect of costimulatory molecules on antigen-specific T cell responses. We show in this section that OX40L and CD70 did not improve Gag-specific T cell responses in SIV-naïve as well as SIV-infected monkeys *in vitro*.

Nada M. Melhem ran all experiments and wrote this chapter. Plasmid generation and real-time PCR were done by Xiang Dong Liu. This study is in partial fulfillment of specific aim 3.

5.2 ABSTRACT

The goal of HIV-1 therapeutic vaccine is to stimulate strong CTL and Th cell responses. DC-based vaccines have been widely studied and we and others reported on the capability of mRNA transfected-DC encoding viral antigen to induce antigen-specific T cell responses *in vitro*. In this report, we wanted to investigate whether we can enhance the immunostimulatory functions of monkey monocyte-derived dendritic cells (DC) propagated from SIV-infected macaques by transfecting cells with one or two mRNA encoding OX40L or CD70, members of the tumor-necrosis factor receptor family (TNFR). We show that the endogenous expression of these molecules is higher in mature DC or DC transfected with *gag* mRNA, as compared to cells in their immature state. When CD40L was immediately added following nucleofection with *gag* mRNA, endogenous expression of OX40L and CD70 was improved. Compared to cells derived from SIV-naïve macaques, DC propagated from SIV-infected animals followed the same trend for endogenous expression of costimulatory molecules but with lower values. We show expression of OX40L and CD70 by western blot analyses following nucleofection of monkey DC with the respective mRNA. In addition, transfection of DC with more than one gene does not impact their translational efficiency. Unexpectedly, our results indicate that DC cotransfected with *gag* mRNA as well as CD70 or OX40L mRNA did not improve the immunostimulatory capability of DC derived from chronically infected macaques. We suggest further *in vitro* investigations to confirm the role of these molecules during chronic SIV infection.

5.3 INTRODUCTION

There is increasing evidence that the tumor necrosis factor receptor family (TNFR), including important costimulatory molecules, have the ability to promote the quality of T cell responses and improve T cell memory (243, 276). This is particularly important when designing a therapeutic vaccine for HIV-1 because the quality and the magnitude of T cell responses are expected to decline with the progress of infection (37). The TNFR family contains several molecules. In this study, we are interested in OX40L and CD70. OX40 (CD134) and CD27 interact with their ligands OX40L and CD70, respectively (55, 276). OX40 is transiently expressed on $CD4^+$ and $CD8^+$ T cells following T cell receptor ligation (41, 253). CD27, the respective receptor of CD70, is expressed on naïve T cells (94). OX40L and CD70 are expressed on activated antigen presenting cells (APC), namely dendritic cells (DC) (55, 243, 276). Upon antigenic challenge, DC, B and T lymphocytes at priming and effector sites, acquire CD70. CD70 expression is transient and associated with the presence of antigen-specific $CD8^+$ T cells (33). It has been reported that OX40L ,either alone or in combination with 4-1BBL, enhances the production of IL-2 and IFN-γ from T cells and stimulates CTL and memory $CD8^+$ T cell responses (59, 239). T cell survival has been enhanced due to increased proliferation and cytokine production as a result of OX40-OX40L signaling (93). The interaction between CD70 and its receptor CD27 on T cells enhances T cell proliferation, cytokine production, as well as cell survival and enhancement of memory T cells (33, 276). Moreover, expression of CD70 on APC is involved in $CD4^+$ T cell help for $CD8^+$ T cell responses (253).

In primary HIV-1 infection, the emergence of CTL is correlated with a dramatic decrease in viral load (31, 120, 136, 235). Later during infection, CTL fail to control viral replication due to escape mutations arising in T cell epitopes as a result of selective pressure *in vivo* (92). The

role of $CD4^+$ T cell responses in the generation as well as the maintenance of effective T cell memory responses has been well documented (117, 240, 252). Moreover, a better $CD4^+$ T cell response can enhance a CTL response (79, 125). Recently, it has been reported that mRNA-transfected DC encoding OX40L enhance the immunostimulatory functions of DC by promoting an Ag-specific $CD4^+$ T cell response, Th-1 polarizing activity as well as Ag-specific CTL responses *in vitro*. In addition, vaccination of mice with cancer using OX40L mRNA-transfected DC resulted in an enhanced antitumor immunity as a result of *in vivo* priming of Th1 T cell responses (59).

Given the involvement of OX40-OX40L and CD70-CD27 signaling in the promotion of human memory T cell functions (285), we sought to investigate the role of these costimulatory molecules in the enhancement of CTL responses *in vitro* in SIV-infected macaques. We have previously demonstrated that mRNA-transfected DC expressing SIV Gag derived from infected macaques stimulate broad and relevant T cell responses. In this study, we evaluated the capacity of *gag* mRNA-transfected DC cotransfected with OX40L mRNA and /or CD70 mRNA to improve the immunostimulatory functions of DC derived from SIV-infected rhesus macaques in an *in vitro* system.

5.4 MATERIALS AND METHODS

5.4.1 Plasmids, mRNA generation

gfp was subcloned from pGEM4Z/GFP/A64 (27) into pSP73-Sph/A64 (183, 184) using BamHI to generate pSP73/GFP/A64. SIVmac239 Gag as well as SIVmac251 Gag were also subcloned into pSP73 and linearization of plasmids encoding GFP or Gag and *in vitro* transcription were performed as before (169).

5.4.2. Cloning and expression of rhesus macaque OX40L and CD70

OX40L and CD70 are known to be induced on mouse splenic DC by exposure to CD40L (45, 201). Thus, for the generation of monkey CD70 and OX40L, $4x10^6$ arcHIV-1ed lymph node cells from SIV-naïve rhesus macaque were cultured in complete DC medium for 24 h in the absence or presence of 3μg/ml CD40L. These lymph node cells were used as a source of RNA for cloning of OX40L and CD70. RNA was extracted using RNeasy kit (Qiagen, Chastworth, CA). Using primers based on the human sequence of CD70 (Gene bank # L08096) and OX40L (Gene bank # X79929), RT-PCR was performed to amplify the monkey homologues. The forward and reverse primers are: OX40LF: 5'CCCTCGAGGCCACCATGGAAAGGGTCCAACCCCTGGAAGAAGAGA; OX40LR: 5'GCCCAAGCTTGCCATCAGCCCCTCAAAGGACAC3'; CD70F: 5'GGAAGCTTGCCACCATGCCGGAGGAGGGTTC3';CD70R:5'CGGAATTCTCAGGGGC GCACCCACTG3'. Using XhoI and HindIII for OX40L and HindIII and EcoRI for CD70, genes were subcloned into pSP73 vector to generate pSP73/OX40L/A64 and pSP73/CD70/A64. Using the following forward and reverse primers, OX40LF and OX40LHAR

(5'GCAAGCTTTCAAGCGTAATCTGGTACGTCGTATGGATAAAGGACACAGA3') as well as CD70F and CD70HAR (5'CGGAATTCTCAAACGTAATCTGGTACGTCGTATGGATAGCGCACCCACTG3'), we amplified OX40L-HA and CD70-HA genes, respectively and then subcloned them into pSP73 for the generation of pSP73/OX40L-HA/A64 and pSP73/CD70-HA/A64. Influenza HA was tagged to CD70 and OX40L to facilitate the detection of expressed proteins. Linearization was performed with Not I and mRNA generation as before (169). At 24 h post transfection with OX40L-HA or CD70-HA mRNA, monkey DC were lysed and lysates separated by gel electrophoresis and respective proteins detected by mAB anti-HA peroxidase (169). The endogenous expression of CD70 and OX40L was performed by real time PCR (Taqman EZRT-PCR Core reagents, Applied Biosystems, Foster City, CA) as per manufacturer's recommendations using the RNeasy kit for RNA extraction, followed by treatment with DNase I (Amplification Grade, Invitrogen, Carlsbad, CA). PDH was used as internal control for real time RT-PCR.

5.4.2 Generation and transfection of DC

Monkey monocyte-derived DC were generated from peripheral blood mononuclear cells (PBMC) and grown in the presence of GM-CSF and IL-4. Day five immature DC were transfected by the amaxa nucleofector with various mRNA. GFP expression was measured in transfected cells by flow cytometry using a Beckman Coulter cytometer (Miami, FL) as previously described (169).

5.4.3 ELISPOT assay

Specific IFN-γ production was detected by ELISPOT assay as described (169). Briefly, DC were washed and incubated with PBMC at a 1:10 ratio in the ELISPOT assay. DC were transfected with mRNA encoding *gag, gag* and CD70*, gag* and OX40L*, gag* and CD70 and OX40L, OX40L and CD70, or *gfp*. Immediately following transfection, DC were matured with CD40L for 24h prior to incubation with PBMC.

5.5 RESULTS

5.5.1 Endogenous expression of CD70 and OX40L in monkey monocyte-derived DC

In preliminary experiments, real-time PCR was performed to determine the endogenous expression of OX40L and CD70 in monkey monocyte-derived DC. This is important to document underrepresented genes and consequently compensate for it by mRNA transfection. For this purpose, DC from SIV-naïve macaques were exposed to treatments and message expression of costimulatory molecules detected. When DC were transfected with *gag* mRNA and immediately matured with CD40L, there was an increase in endogenous expression of CD70 compared to immature cells treated similarly (Figure 12A). This expression was comparable to what we observed in *gag* mRNA-transfected DC treated immediately with TLR8 agonist , which synergize with TLR8 to induce DC maturation (data not shown) (187). When DC were cotransfected with *gag* mRNA and CD70 mRNA and immediately matured with CD40L, it was evident that we have successfully introduced the CD70 gene into these cells as indicated by the

high increase in fold change above immature DC (data not shown). We observed a similar trend for the endogenous expression of OX40L in DC propagated from SIV-naïve macaques (Figure 12B). We sought to perform western blot analyses on DC treated as above and using anti-human CD27L Ab (R & D systems) and anti-human OX40L Ab (R & D systems) to detect endogenous expression of OX40L and CD70 . We were not able to detect protein expression (data not shown).

We performed similar experiments using DC propagated from blood of SIV-infected macaques. We found similar pattern as those described above; however, the levels of OX40L and CD70 endogenous expression in DC were lower (Figure 13). Consequently, we expected that transfection of DC with mRNA encoding one or both costimualtory molecules, would lead to increased protein expression and thus might add beneficial effects for T cell stimulation.

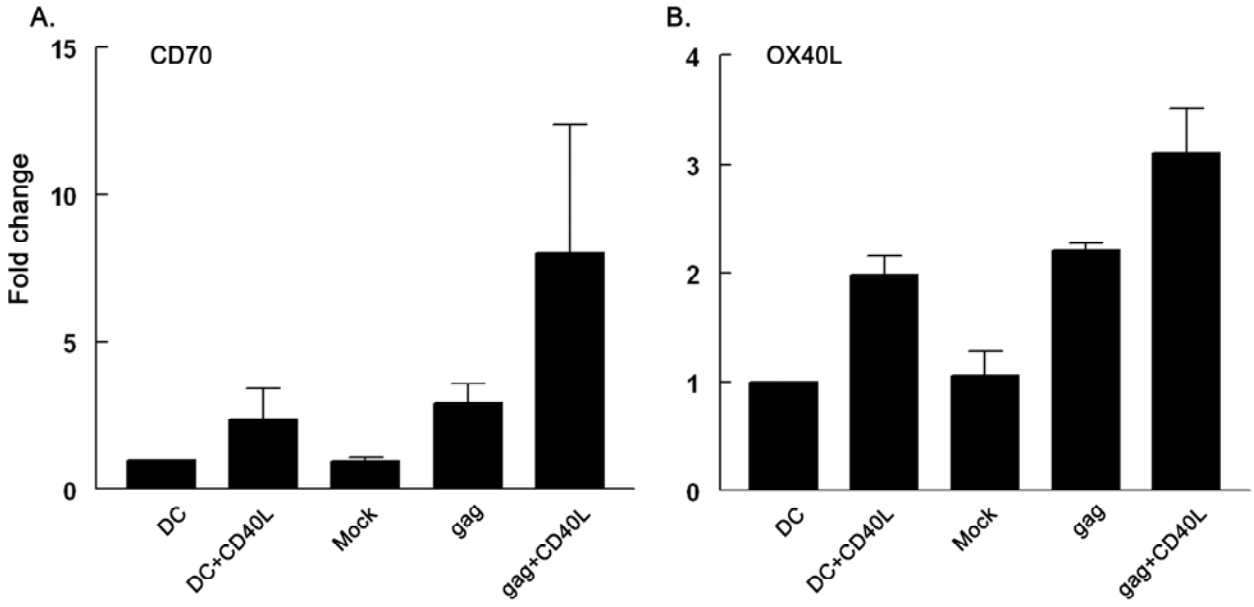

Figure 12. Endogenous expression of CD70 and OX40L in monkey monocyte-derived DC isolated from SIV-naïve macaques. CD70 (A) and OX40L (B) message expression was determined by real-time PCR in monocyte-derived DC propagated from blood of SIV-naïve macaques. The conditions are the following: immature DC (DC), DC matured with CD40L (DC+CD40L), mock-nucleofected DC (mock), SIVmac239 *gag* mRNA-transfected DC (*gag)*, SIVmac239 *gag* mRNA transfected DC and matured with CD40L (*gag* +CD40L). Results are representative of two experiments.

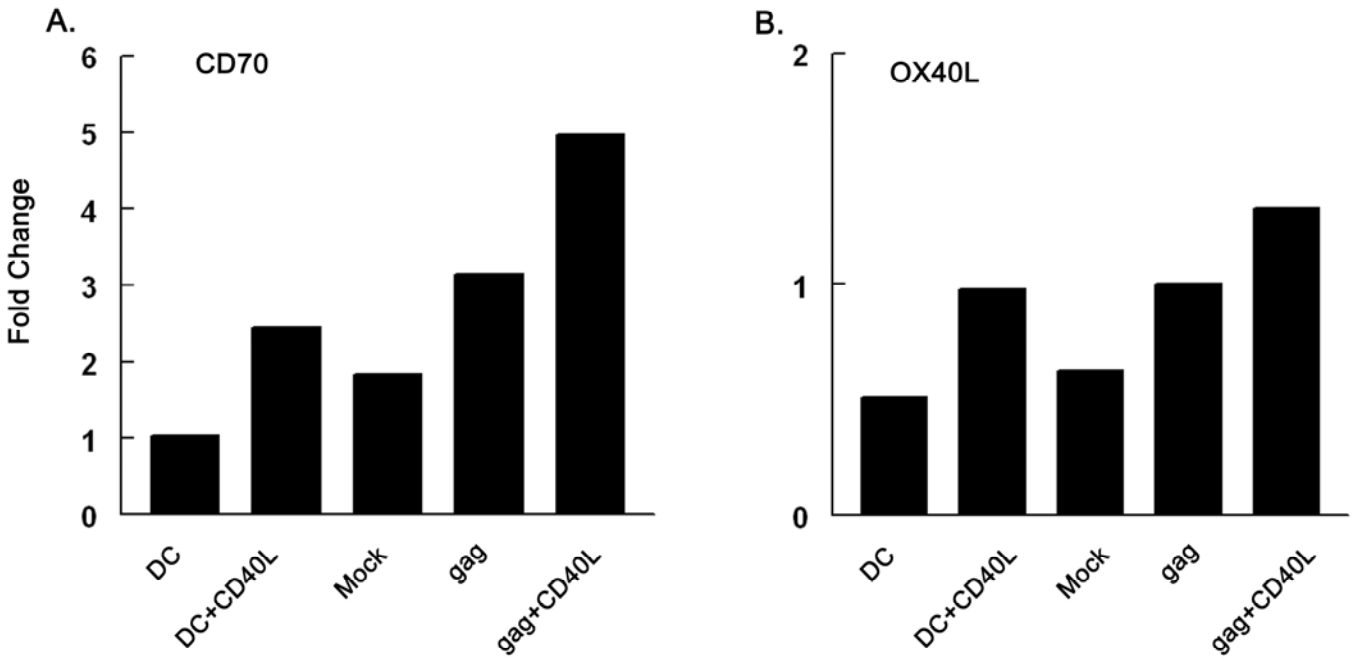

Figure 13. Endogenous expression of CD70 and OX40L in monkey monocyte-derived DC isolated from SIV-infected monkeys. CD70 (A) and OX40L (B) message expression was determined by real-time PCR in monocyte-derived DC propagated from blood of SIVmac251-infected macaques. The conditions are the following: immature DC (DC), DC matured with CD40L (DC+CD40L), mock-nucleofected DC (mock), SIVmac251 *gag* mRNA-transfected DC (*gag)*, SIVmac251 *gag* mRNA transfected DC and matured with CD40L (*gag* +CD40L).

5.5.2 Nucleofection of DC with CD70 or OX40L mRNA results in protein expression

Monkey CD70 and OX40L genes subcloned into pSP73-Sph/A64 were used as templates for DNA linearization followed by *in vitro* transcription for the generation of the respective mRNA (Figure 14A). Monkey monocyte-derived DC were nucleofected with OX40L or CD70 mRNA. 24h post-transfection, proteins were detected in cell lysates following probing with an HA-specific mAB since CD70 and OX40L were linked to influenza hemagglutinin (Figure 14B). This confirms that mRNA encoding costimulatory molecules introduced into DC is successfully translated.

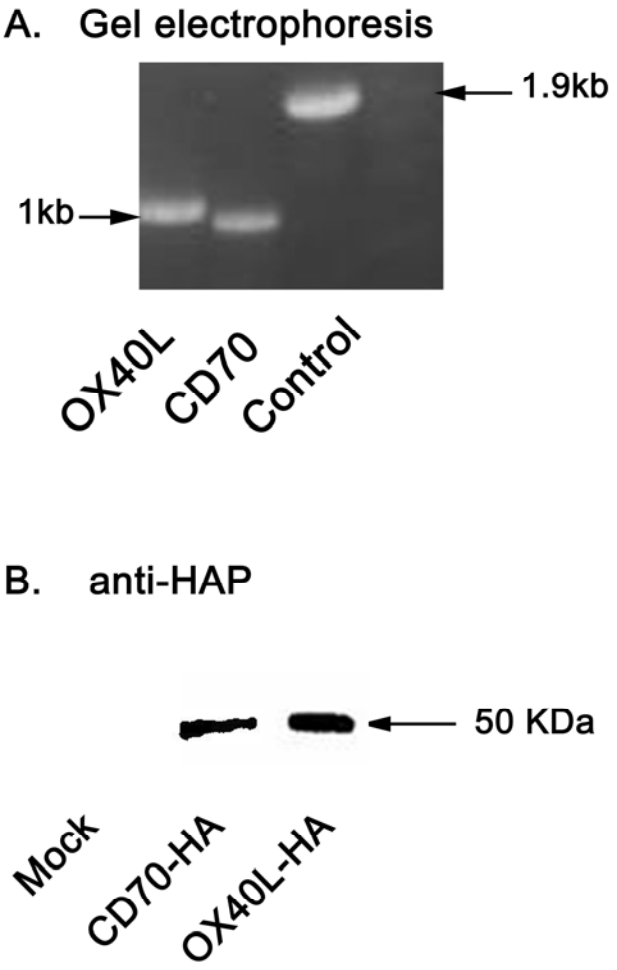

Figure 14. Nucleofection of monkey monocyte-derived DC with OX40L or CD70 mRNA results in protein expression.(A) Not 1 linearized pSP73/OX40L-HA/A64 or pSP73/CD70-HA/A64 plasmids were used for generation of mRNA by *in vitro* transcription. (B) Immature monkey monocyte-derived DC were nucleofected with OX40L-HA mRNA, CD70-HA mRNA or mock-nucleofected and cell lysates probed with anti-HA peroxidase mAb 24 hours later.

5.5.3 Transfection efficiency is not affected by cotransfection of monkey monocyte-derived DC with multiple genes

The advantage of using DC-based immunotherapy is that multiple immunogenic genes could be administered to these cells, simultaneously for the stimulation of multispecific T cell responses. We investigated the possibility of administering two mRNA to monkey monocyte-derived DC by nucleofection. DC were cotransfected with mRNA encoding Gag and another one encoding a reporter gene, GFP. 24h post transfection, flow cytometry was performed to detect protein expression. The transfection efficiency of DC, as detected by GFP production was not changed as compared to cells transfected with *gfp* mRNA only (Figure 15).

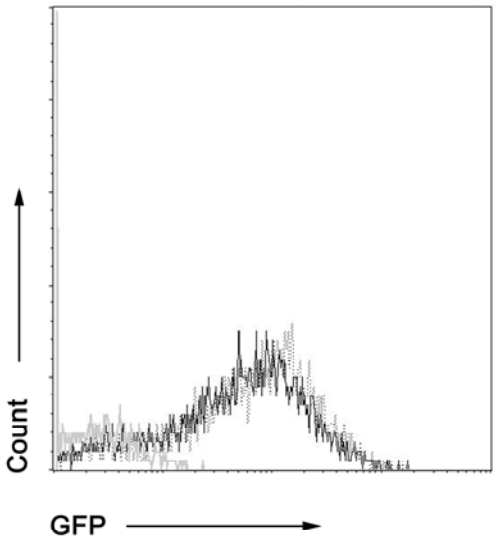

Figure 15. Cotransfection of monkey monocyte-derived DC with *gag* and/or *gfp* mRNA does not impact transfection efficiency of the reporter gene. Monkey monocyte-derived DC were transfected with mRNA encoding *GFP* or with *gfp* mRNA and SIVmac239 *gag* mRNA. 24h post transfection, flow cytometry was performed to detect GFP expression. Results are representative of two experiments. Bold line: GFP, dotted line: GFP and Gag, gray line: mock-nucleofected DC.

5.5.4 Costimulatory TNFR molecules do not enhance specific CTL responses

We have previously demonstrated that *gag* mRNA-transfected DC induce potent and specific T cell responses *in vitro* in SIV-naïve and SIV-infected macaques (169) (Chapters 1 & 2). In this study, we evaluated whether cotransfection with mRNA encoding costimulatory molecules along with SIV Ag would induce better specific CTL responses. IFN-γ ELISPOT assay was used to determine the overall response to *gag* mRNA in the presence and absence of costimulatory molecules. Monkey monocyte-derived DC propagated from blood of a healthy macaque were nucleofected with *gag* mRNA, cotransfected with *gag* mRNA and CD70 or OX40L mRNA or cotransfected with three mRNA encoding *gag*, CD70 and OX40L. The level of Gag-specific T cell reactivity to *gag* mRNA was the same regardless of the presence or absence of OX40L and/or CD70 (Figure 16). The value of using these costimulatory molecules is to determine their impact on T cell responses particularly during chronic SIV infections. Thus, DC propagated from blood of two monkeys (R484 and R486), infected with SIVmac251, were transfected with *gag* mRNA, cotransfected with *gag* and CD70 or OX40L mRNA or cotransfected with mRNA encoding all three genes. The latter was performed with cells from R484 but not R486 due to insufficient cell numbers. IFN-γ ELISPOT assays were performed. The magnitude of Gag-specific T cell responses was almost the same in the absence or presence of costimulatory molecules (Figure 16). In addition, non-specific T cell stimulation was observed when DC were transfected with *gfp* mRNA or CD70 and OX40L mRNA. In our hands, OX40L and CD70 did not enhance better T cell responses *in vitro*.

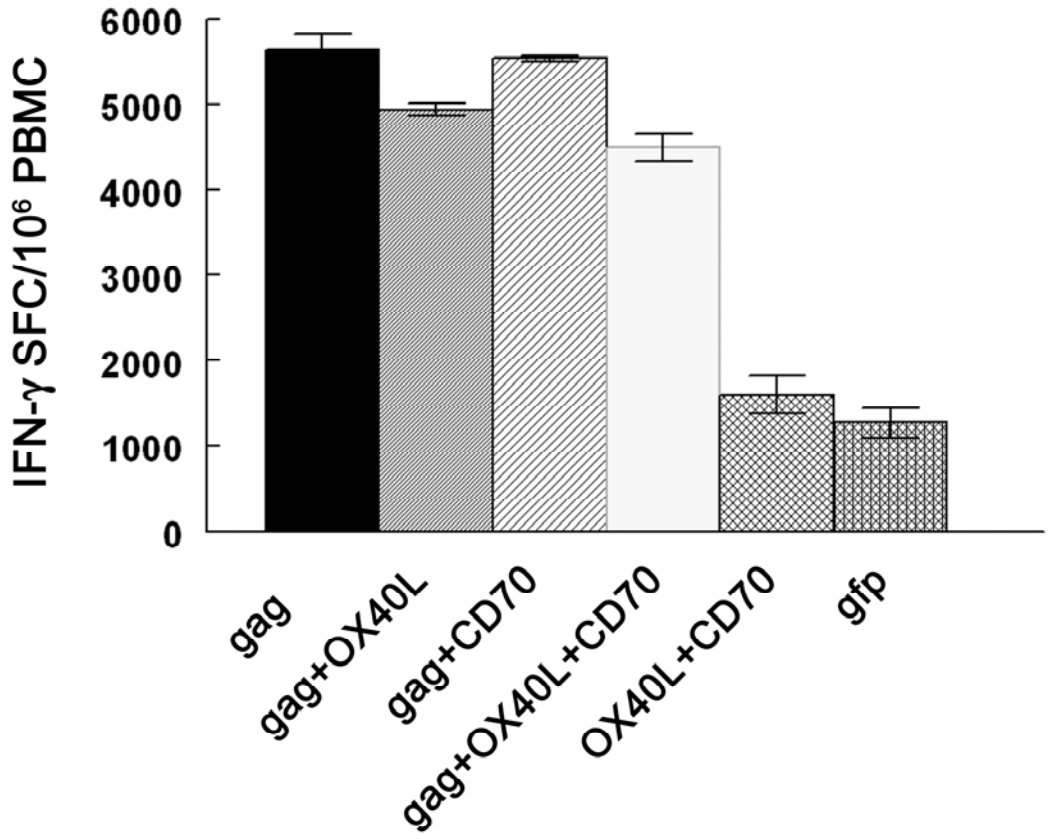

Figure 16. Immunostimulatory functions of DC propagated from SIV-naïve macaques are not promoted by TNFR costimulatory molecules *in vitro*. Immature DC propagated from a healthy macaque (R187) were transfected with the following mRNA: *gag*, *gag* and OX40L, *gag* and CD70, *gag*, OX40L, and CD70, OX40L and CD70; or *gfp*. DC were used as stimulators of autologous PBMC for 24h in an IFN-γ ELISPOT assay. Shown are mean ±SEM of triplicate determinations. SFC=spot-forming cells. *gag* mRNA used encodes SIVmac239 Gag.

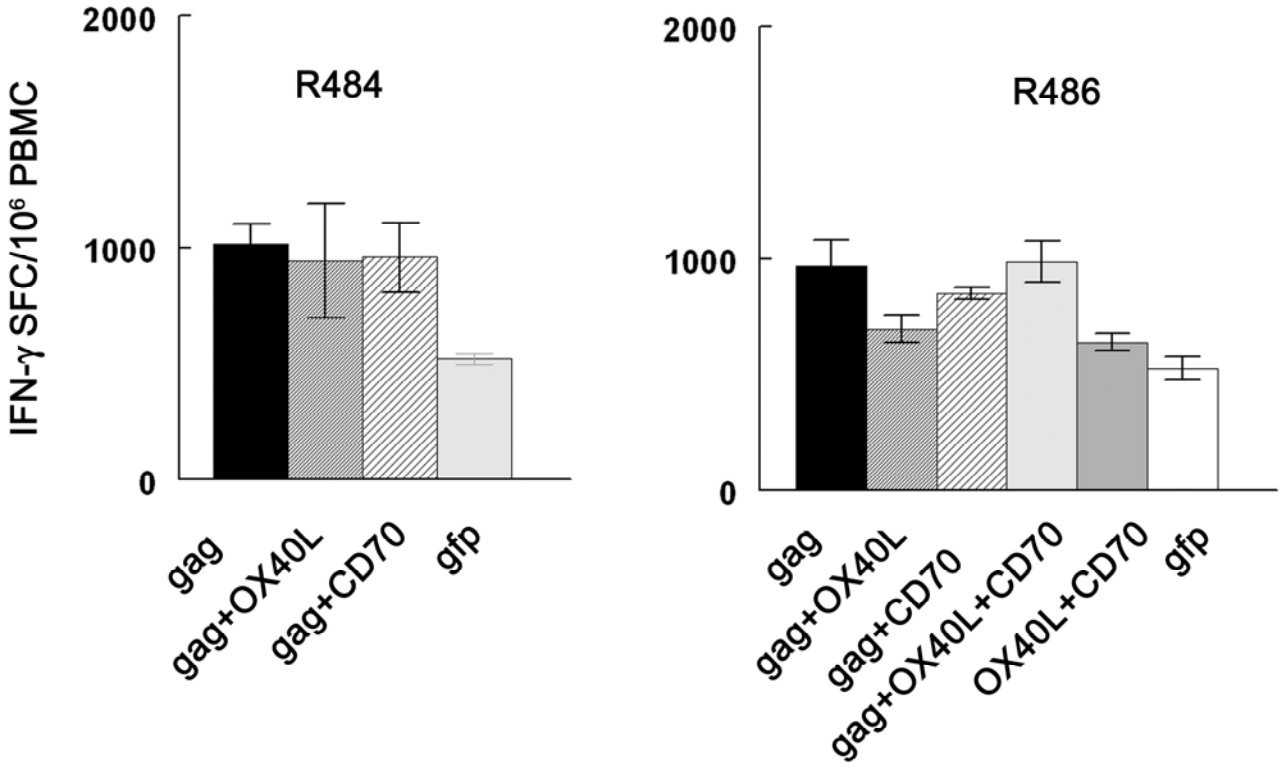

Figure 17. Immunostimulatory functions of DC propagated from SIV-infected macaques are not promoted by TNFR costimulatory molecules *in vitro*. Immature DC propagated from two rhesus macaques infected with SIVmac251 (R484, R486) were transfected with *gag*, *gag* and OX40L, *gag* and CD70, *gag*, OX40L and CD70 (R484), OX40L and CD70; or *gfp*. DC from the R486 were not cotransfected with gag, CD70 and OX40L mRNA, simultaneously due to insufficient numbers of harvested DC. DC were used as stimulators of autologous PBMC for 24h in an IFN-γ ELISPOT assay. Shown are mean ±SEM of triplicate determinations. SFC=spot-forming cells. *gag* mRNA encodes SIVmac251 Gag.

5.6 DISCUSSION

The goal of any vaccine strategy directed against HIV-1/AIDS is the generation of a strong cell mediated immune response capable of controlling virus replication. DC are the most potent antigen presenting cells. Immature DC acquire antigen from peripheral tissues and then migrate to the lymph nodes, a process during which they undergo maturation as well as phenotypic changes (16). The use of DC in the design and development of tumor as well HIV-1 vaccines is being extensively studied (86, 275). Based on observations made by us (169) and others (129, 266, 269), transfection of DC with SIV or HIV-1 mRNA is successful at generating strong Ag-specific T cell responses. Here we sought to determine the impact of cotransfection of DC with costimulatory molecules on T cell responses along with virus Ag.

Low OX40L cell-surface expression was observed on human monocyte-derived DC in their immature state as well as when matured with TNF-α, IL-1β, IL-6 and PGE2 (59). However, CD40L-matured DC induced OX40L expression. Moreover, authors were able to overcome low OX40L surface expression on DC by transfection with OX40L mRNA. Our results indicate that the endogenous message expression of CD70 and OX40L is higher in DC propagated from blood of SIV-naïve and SIV-infected and matured with CD40L as compared to immature cells. This level is similar to the one observed after transfection of DC with mRNA encoding wild-type Gag and an improvement in expression is observed when *gag* mRNA transfected DC are exposed to CD40L. This agrees with our previous data showing that mRNA results in modest DC maturation and that addition of CD40L provides additional benefits at maturation (169) as well as endogenous expression of costimulatory molecules.

In this report, we show that transfection of monkey monocyte-derived DC with more than one *in vitro* transcribed mRNA does not negatively affect expression of the marker gene GFP. This is critical since we are suggesting the introduction of multiple genes into monocyte-derived DC. We have shown significant T cell responses to DC transfected with mRNA encoding autologous Gag sequences isolated from SIV-infected macaques. However, these responses were lower than those observed in healthy macaques (169). This could be due to weak T cell responses, reported in HIV-1-infected individuals and SIV-infected monkeys (11, 100, 172, 284). Following confirmation of expression of CD70 and OX40L in mRNA-transfected DC by western blot, as we successfully demonstrated Gag expression in monkey monocyte-derived transfected with wild-type *gag* mRNA (169), we next assessed whether DC transfected with costimulatory molecules are capable of improving Ag-specific $CD8^+$ T cell responses. Surprisingly, our *in vitro* data shows that viral specific CTL responses are not enhanced by cotransfection of DC with CD70 and/or OX40L mRNA along with *gag* mRNA when DC originated from both SIV-naïve or infected monkeys. These results are contradictory with previous reports demonstrating *in vitro* improvement of CTL and $CD4^+$ T cell responses (59).

Due to the loss of $CD4^+$ memory T cells from the mucosa and other tissues during SIV and HIV-1 infections (34, 151, 165, 209), we suggest the reassessment of the role of these costimulatory molecules for the enhancement of viral specific T cell responses, particularly in chronically infected macaques with low $CD4^+$ counts. Moreover, we previously reported that mRNA transfected DC encoding wild-type Gag bearing the targeting signal of the lysosomal associated membrane protein-1 (LAMP-1) leads to strong $CD8^+$ T cell responses and enhances $CD4^+$ T cell responses *in vitro* (169). Consequently, cotransfection of DC with mRNA encoding virus-specific antigen and bearing LAMP-1 targeting signal and as well as mRNA encoding

these costimulatory molecules might highlight the importance of costimulatory molecules towards enhancing Ag-specific $CD8^+$ and promoting $CD4^+$ T cell responses.

Acknowledgements. This work was supported by National Institutes of Health grant AI52052 to S.M. Barratt-Boyes. We would like to thank Xiang Dong Liu for assistance with this work as well as Nicole Banichar for animal procedures.

6.0 OVERALL SUMMARY AND DISCUSSION

Despite the fact that ART can control HIV-1 infection by reducing viral replication in $CD4^+$ T cells as well as other cell types in blood and lymphoid tissues, the persistence of reservoirs of the virus and the emergence of viral variants represent important obstacles in the treatment of HIV-1 (92, 149).Thus, the development of HIV-1 therapeutic vaccine is urgently needed. The first step in any vaccine design is to deliver the antigen in a way leading to the generation of efficient and strong T cell responses. DC are the most potent antigen-presenting cells. Immature DC uptake and process antigen, a process leading to their maturation and consequent migration to the lymph nodes (16, 231). Reaching the lymph nodes, DC attract and activate T cells (232). This unique property made DC a subject of interest for the generation of cancer and HIV-1 vaccines (86, 275).

The current project aimed to: (1) determine the best transfection method for antigen delivery to monocyte-derived DC; (2) investigate the ability of monkey monocyte-derived DC to induce specific T cell responses against autologous virus sequences isolated during SIV infection; and (3) determine whether costimulatory molecules like OX40L and CD70 can improve the immunostimulatory capacity of antigen-transfected monkey monocyte-derived DC.

6.1 MRNA IS BETTER THAN DNA FOR STIMULATION OF ANTIGEN-SPECIFIC T CELL RESPONSES

Prior work had described the use of plasmid DNA as well as mRNA as antigen sources for transfection of DC and their potential use as therapeutic agents against cancer and infectious agents (86, 102, 129, 169, 212, 248, 257, 266, 269). These alternatives developed due to the disadvantages associated with the use of viral vectors, considered as preferred methods for antigen delivery to DC. Viral vectors based on adenovirus, poxviruses and lentivirus induce high level antigen expression but might lead to undesired vector –specific immune responses (72, 118, 176, 292). A common finding in studies using mRNA or DNA, is that human monocyte-derived DC transfected with antigens encoded by *in vitro* transcribed mRNA are able of inducing antigen-specific T cell responses despite the use of different delivery methods. In an effort to learn more about the use of mRNA encoding autologous virus sequences as a therapeutic approach for the treatment of HIV-1, we used the rhesus macaque SIV model to test the potential of DC-based vaccines using virus derived sequences.

6.1.1 Transfection efficiency of monocyte-derived DC with DNA and mRNA

Our first goal was to address the efficacy of transfecting monkey monocyte-derived DC and to compare the transfection efficiency of DC with plasmid DNA and mRNA. In preliminary experiments, we used leukemic K562 cells, human monocyte-derived DC along with monkey monocyte-derived DC ; these cells were transfected with DNA or mRNA encoding GFP and a side-by-side comparison was performed following use of Transfast^{TM}, Transmessenger reagent, electroporation and nucleofection. We found that nucleofection is a superior method for delivery

of both DNA and mRNA. However, mRNA delivery into DC *via* nucleofection maintains relatively high viability and consistently produces higher protein levels in transfected cells as compared to DNA-transfected DC. Our results are consistent with previous findings in primary rat neurons and murine macrophage cell lines (82, 265) as well as human monocyte-derived DC (140). We report that mRNA is a highly efficacious method for the introduction of transgenes into DC associated with sustained antigen expression (Chapter 1). These findings are not surprising particularly in non-dividing cells like DC especially since DNA undergoes limited trafficking to the nucleus (161). In contrast, mRNA is immediately translated in the cytoplasm and does not pose a risk like DNA integration in the cellular genetic material. Entry of plasmid DNA into the cell is a multistep process starting by internalization into the cytoplasm in the presence of cytoplasmic enzymes. The results of the competition between the DNA entry as well as intracellular degradation determines gene expression (161, 258).Thus DNA is at risk during the loading process into cells.

We found that immature DC as well as DC matured with CD40L immediately post nucleofection yield similar transfection efficiencies. This was observed following DNA as well as mRNA nucleofection (Chapter 1). In our hands, protein expression post transfection with nucleic acids is not affected by the DC state. Our results are consistent with previous reports using electroporation for transgene expression (146, 171). Other reports showed that DNA transfection of murine bone marrow derived DC is more effective following CD40-mediated maturation of (142). Moreover, we show that human monocyte-derived DC transfected with DNA are refractory to maturation with CD40L as is the case with lipopolysaccharide (146). These effects could be due to the toxicity exerted by DNA on DC leading subsequently to lower levels of protein expression in viable cells.

6.1.2 Nucleofection of Human and monkey monocyte-derived DC with mRNA induces phenotypic maturation

An important factor in DC-based immunotherapy is the degree of cell maturation for optimal antigen presentation especially since immature DC capture antigen but mature DC process and present it to naïve T cells (16, 231). We sought to analyze the effect of mRNA and DNA nucleofection on maturation of human monocyte-derived DC as there have been contradicting reports depending on the transfection system used (43, 103, 128, 140, 169, 248, 269, 279). Our data indicates that mRNA transfection induces DC maturation whereas DNA limits the capacity of DC to undergo maturation (Chapter 1). In addition, nucleofection of monkey monocyte-derived DC with mRNA also induced phenotypic maturation (169) (Chapter 2). In both cases, the addition of CD40L provided additional benefits reflected by more pronounced shift in expression of costimulatory molecules. *In vitro* transcribed mRNA is reported to stimulate TLR3 on DC and induce immune activation likely through secondary structures (43). Thus, mRNA is an endogenous ligand for TLR3 (128). Pathogens escaping immune surveillance also escape detection by TLRs (192). Consequently, loading DC with mRNA, particularly autologous mRNA isolated from infected individuals at the time of immunotherapy, might enhance their ability to activate the immune system in the face of an escape event. Moreover, ligation of TLR3 and TLR8 results in maturation of myeloid DC. Thus mRNA might synergize with agonists of other TLR, i.e. TLR8, to activate DC and induce higher production of IL-12 and IL-23 (187).

6.1.3 Monkey monocyte-derived DC nucleofected with wild-type *gag* mRNA but not DNA induce efficient and specific T cell stimulation

We next sought to perform functional studies to investigate the impact of nucleofection of DC with wild-type non codon-optimized mRNA or DNA on the capacity of transfected cells to induce antigen-specific T cell responses using the rhesus macaque model. This monkey model provides an ideal preclinical setting to test the therapeutic potential of DC-based vaccines. We found that DC nucleofected with mRNA encoding wild-type SIVmac239 Gag induced antigen-specific CTL responses *in vitro* whereas DNA did not. Our results are consistent with previous reports (248, 269).

To our knowledge, this is the first report comparing the capacity of stimulating specific T cell responses *in vitro* following nucleofection of DC with mRNA and DNA encoding virus-specific antigen. We speculated to observe lower CTL responses induced by plasmid DNA-transfected DC due to the fact that mRNA is immediately translated into protein inside the cytoplasm whereas DNA has to be transcribed, translated into proteins and subsequently peptides derived from the translated proteins presented to T cells *via* MHC class I molecules. It was unexpected that plasmid DNA does not stimulate specific-T cell responses. The results summarized above were observed at 24 and 48 h post nucleofection of DC with mRNA or plasmid DNA encoding Gag. These results coincide with the long lived gene expression (Chapter 1) in DC, thus an extended supply of antigen for presentation following transfection with mRNA. Other studies reported similar findings following transfection of human monocyte-derived DC with mRNA using electroporation or nucleofection (140, 169, 263, 269). Our results confirm the usefulness of mRNA-based delivery of genes in retaining DC viability and responsiveness to

maturation stimuli that are important for T-cell stimulation, whereas DNA delivery could have deleterious effects on the functional properties of DC.

In the field of HIV-1, DC transfection was performed using mRNA encoding codon-optimized antigens to stimulate antigen-specific T cell responses in vitro (266, 279). Although codon optimization allows enhanced gene expression and make these genes promising vaccine candidates, these sequences do not reflect the circulating virus sequences in an infected individual. Moreover, with our optimized nucleofection system, protein expression in DC transfected with mRNA encoding wild-type Gag is consistently high (Chapters 1 and 2).

Our protocol for T cell assays follows the sequential addition of a maturation stimulus, CD40L, immediately post transfection with mRNA. Specific *in vitro* T cell responses to viral antigen were detected against mRNA-transfected DC propagated from SIV-naïve as well as SIV-positive macaques (Chapter 2). This coincides with higher expression of costimulatory molecules on DC (Chapters 1 and 2) or perhaps a cytokine profile enhanced by addition of CD40L. Our results are similar to previous reports, emphasizing the use of different maturation stimuli for better T cell stimulatory activity (123, 152, 178, 211, 234, 263). We believe that mRNA transfection of DC followed by maturation is a logical sequence especially if intranodal administration of the vaccine is to be performed for *in vivo* studies.

6.2 AUTOLOGOUS VERSUS HETEROLOGOUS VIRUS-DERIVED SEQUENCES FOR T CELL STIMULATION

DC-based HIV-1/SIV vaccines described in the literature are based on well characterized strains of virus (35, 155, 157, 189, 273). This approach may not be suitable for therapeutic vaccination since epitopes expressed by DC may not reflect those present in the infected individual at the time of immunotherapy due to the ability of the virus to undergo escape mutations in the T-cell epitopes through selective pressure *in vivo* (92). The present study provides evidence supporting the use of mRNA encoding autologous virus sequences isolated from SIV-infected macaques for the generation of relevant antigen-specific T cell responses. The following briefly summarizes our findings:

6.2.1 SIV infection does not affect the capacity of DC to be transfected

Given that our goal is to use mRNA-nucleofected DC during SIV infection, we evaluated whether SIV infection affects the transfection efficiency of DC. Our data show no evidence of reduction in mRNA transfection efficiency in DC isolated from SIV-naïve, SIV-infected or monkey with neuroAIDS (Chapter 2).

6.2.2 The effect of diverting antigen into the lysosomal/endosomal pathway

There is extensive evidence that cellular immunity plays an important role in control of HIV-1 infection and that virus-specific $CD4^+$ T cell responses are critical in maintenance of CTL (205, 223, 227). Taken together, these findings support the development of therapeutic immunization

strategies stimulating both $CD4^+$ and $CD8^+$ T cell responses. We are proposing the use of mRNA as a source of antigen loaded onto DC as an approach for therapeutic HIV-1 vaccine. This mRNA is translated endogenously and subsequently, the respective protein is degraded by the proteasome and enter the MHC class I pathway. We generated mRNA encoding wild-type non codon optimized Gag bearing the targeting signal of the lysosomal associated membrane protein-1 (LAMP-1) to induce both antigen-specific $CD8^+$ and $CD4^+$ T cell responses by directing antigen expression to MHC class II molecules. Previous studies reported the enhancement of $CD4^+$ T cell responses (61, 129, 163, 169, 226, 251) whereas others did not find any effect exerted by LAMP-1 when linked to viral or tumor antigens (272). We found that DC from SIV-naïve macaques nucleofected with *gag-LAMP-1* mRNA induced strong $CD8^+$ T cell mediated responses similar to those observed in DC transfected with *gag* mRNA. However, a greater $CD4^+$ T cell responses were induced by the former (Chapter 2).

Our data confirm that DC nucleofected with wild-type *gag* mRNA efficiently present antigen to virus-specific T cells and that channeling Gag antigen to the lysosomal pathway enhances $CD4^+$ T cell responses.

6.2.3 Efficient T cell stimulation using DC transfected with *gag* mRNA encoding a CTL escape mutation

The main purpose of this project is to demonstrate that antigen specific T cell responses are induced by DC transfected with autologous virus sequences isolated from infected macaques at the time of immunotherapy. This goal is particularly important in the face of virus escape. Consequently, we nucleofected DC with mRNA encoding mRNA encoding Gag isolated from a Mamu-A*01-expressing macaque containing a threonine to isoleucine escape mutation at amino

acid 182 within the immunodominant CM9 epitope. The latter induced reduced T cell responses as compared to DC nucleofected with mRNA encoding intact Gag sequences in a non-autologous system.

6.2.4 T cell stimulation by *gag* mRNA transfected-DC in the face of virus escape is directed against subdominant epitopes

We confirmed that the decline of T cell responses mentioned above was due to escape in the CM9 epitope and that the generated response was against intact subdominant epitopes known to be important in virus control (169). This was observed following nucleofection of DC with shorter sequences of mRNA encoding Gag from the inoculum as well as escape viruses (Chapter 2).

6.2.5 Effect of LAMP-1 on the generated T cell responses to mutant Gag

We found that DC nucleofected with *gag*-LAMP-1 mRNA containing an escape mutation in the CM9 immunodominant epitope were able to stimulate almost equivalent $CD8^+$ and $CD4^+$ T cell responses. Consequently, the effect of targeting antigen to the lysosome resulted in an amplified $CD4^+$ T cell responses with the loss of an immunodominant CTL epitope naturally acquired during SIV infection (Chapter 2). These data support the use of lysosome targeted viral antigen in DC-based therapeutic vaccination for HIV-1.

6.2.6 Efficient T cell stimulation by autologous *gag* mRNA transfected-DC

We next generated autologous Gag sequences isolated from chronically infected macaques with SIVmac251 or SIV/DeltaB670 to test the ability of mRNA-transfected DC to stimulate virus-specific T cell responses in an entirely autologous system. As expected, we observed relevant and potent autologous Gag-specific CTL responses (Chapter 2). Our results are consistent with previous findings in HIV-1-infected individuals (266). The value of using autologous sequences is appreciated in the event of a virus escape. This is important since as we have shown, DC transfected with mRNA encoding CTL escape mutations still stimulate relevant and significant specific T cell responses to subdominant epitopes. Such epitopes were reported to be important to *in vivo* HIV-1 control (76). Authors found that high allele frequencies could eliminate subdominant epitopes despite being effective at stimulating CTL responses. Moreover, high affinity CTL down-modulate peptide-MHC complexes on antigen-presenting cells leading to preferential expansion of high-affinity CTL (204). This could be faced when using centralized sequences. In addition, the recent description of the de-novo generation of novel CTL epitopes from mutant immunodominant epitopes (6) highlight the importance of using autologous sequences as these novel epitopes would not be presented by DC transfected with centralized sequences.

In conclusion, HIV-1-1 vaccine based on a single virus sequence, particularly immunodominant, will induce CTL responses that are too specific to provide protection against the quasispecies of the variable HIV-1-1, hence limiting the ability of this vaccine to control the virus. Moreover, inducing CTL responses to epitopes that are no longer present is redundant and may be deleterious in stimulating immunodominant responses that are not capable of recognizing the virus (147). The evolution of HIV-1 is of critical value to HIV-1 vaccine design. To account

for these complexities, a therapeutic vaccine should be tailored towards the autologous virus present in an individual at the time of immunotherapy and thus dominant and subdominant epitopes would be introduced.

6.2.7 Efficient T cell stimulation by autologous *env* mRNA transfected-DC

A vaccine should aim at inducing a multispecific T cell stimulation. Hence, we performed *in vitro* studies using DC nucleofected with autologous *env* mRNA isolated from two SIV-infected macaques at week 25 post-infection. Similarly to our results with Gag-transfected DC, we observed Env-specific T cell responses in an entirely autologous system (Figure 18).

Consequently our system consisting of nucleofection of DC with mRNA encoding autologous virus sequence isolated during the course of infection proves again to be able to stimulate strong specific T cell responses and thus further supporting the usefulness of this model for the development of a therapeutic HIV-1 vaccine.

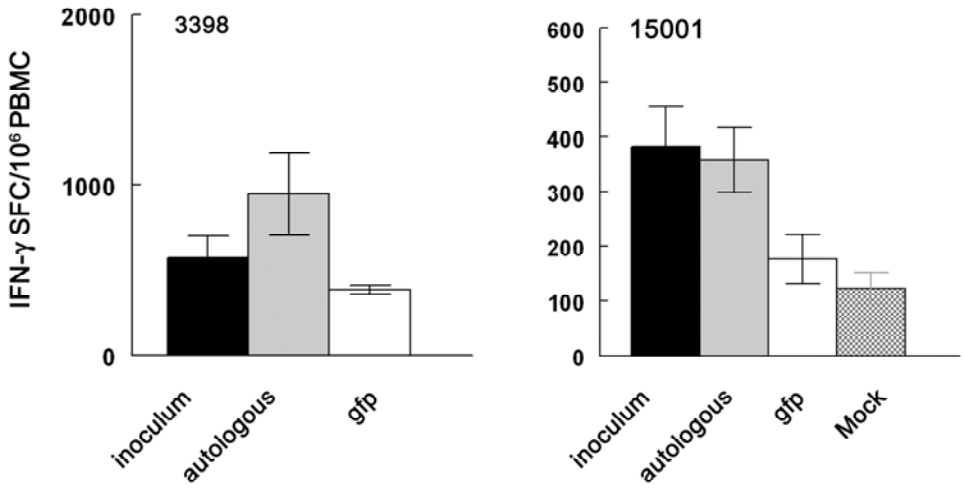

Figure 18. DC nucleofected with *env* mRNA isolated from infected monkeys induce significant Env-specific T-cell responses from autologous PBMC. Immature DC propagated from two rhesus macaques (3398 and 15001) infected with SIV/DeltaB670 were nucleofected with mRNA encoding original inoculum *env*, autologous *env* (week 25 post-infection) *gfp* or mock-nucleofected (one animal) and used as stimulators of autologous PBMC in an IFN-γ ELISPOT assay. DC to PBMC ratio used was 1:10. Shown are mean responses of triplicate determinations with the mean for the group represented by a horizontal line. SFC=spot-forming cells.

6.3 EFFECT OF COSTIMULATORY MOLECULES OX40L AND CD70 ON THE IMMUNOSTIMULATORY CAPACITY OF *GAG* MRNA TRANSFECTED-DC

There is increasing evidence that members of the TNFR family promote the quality of T cell responses as well as improve T cell memory (243, 276). In addition, recent reports demonstrated that DC transfected with mRNA encoding OX40L enhances the immunostimulatory functions of DC *in vitro* as well as lead to enhanced antitumor immunity *in vivo* (59). Consequently, we sought to determine the effect of *gag* mRNA transfected-DC cotransfected with mRNA encoding OX40L, CD70 or both on antigen specific T cell responses. The following is a brief summary of our findings:

6.3.1 The immunostimulatory capacity of *gag* mRNA transfected DC is not enhanced by cotransfection with OX40L and /or CD70 mRNA

We found that DC propagated from SIV-naïve macaques are successfully transfected with more than one gene. This is important as we are proposing the administration of multiple immunogenic genes for the development of an effective HIV-1 vaccine. The results from our studies made us reflect on the possibility of improving the immunostimulatory capacity of mRNA-transfected monocyte-derived DC during SIV infection since we have observed a decline in T cell responses directed against autologous sequences and also due to reports of loss of $CD4^+$ memory T cells from the mucosa and other tissues during SIV and HIV-1 infections (34, 151, 165, 209). Moreover, previous reports demonstrated that CD70 is overexpressed in HIV-1-infected individuals and contributes to better T cell functions compensating for decline in $CD4^+$ help for maintenance of $CD8^+$ T cell responses (197).

Unexpectedly, we found that DC cotransfected with *gag* mRNA as well as mRNA encoding CD70 or OX40L or both, did not improve the immunostimulatory capability of DC derived from SIV-naïve or chronically infected macaques (Chapter 3). It might be that our optimized system with *gag* mRNA is the ultimate level of antigen-specific T cell stimulation. Additional analyses are needed for the elucidation of this matter.

6.4 PUBLIC HEALTH SIGNIFICANCE

Worldwide, 40 million people are living with HIV-1, 4.3 millions were newly infected with the virus in year 2006 and last but not least , 2.9 millions died of AIDS last year (UNAIDS/WHO 2006). Despite the effort and the promising developments to address the AIDS epidemic, the number of people living with HIV-1/AIDS is growing. In spite of the benefits associated with antiretroviral therapy in the longer survival of infected individuals, there is a risk of treatment failure due to viral drug resistance or drug toxicity. Although there was a decline in HIV-1 prevalence in some sub-Saharan African countries, this trend does not have an impact on the global AIDS epidemic. The AIDS epidemic is associated with loss of lives, lower economic productivity as well as higher health costs. Thus, the development of therapeutic HIV-1 vaccine is needed now more than ever, a vaccine that would boost the immune response and contain the virus and as a result decrease its rate of transmission. Given the diversity of clinical isolates (83) and the ability of the virus to undergo CTL escape mutations (92) we have to tailor a vaccine directed towards the autologous virus present in the individual at the time of immunotherapy. Our vaccine design account for this complexity by using DC transfected with mRNA encoding viral sequences reflecting those expressed in the infected macaque. These cells stimulated broad

and relevant T cell responses, the ultimate goal of any vaccine. We believe that taking our design to the *in vivo* model would have a potent protective effect on the immune response and consequently decrease the burdens associated with the infection.

Together, these data support our hypothesis that autologous viral antigen is capable of stimulating relevant and specific T cell responses against the infecting strain at the time of immunotherapy. mRNA-based DC vaccination is a safe and clinically feasible method. Moreover, our data highlight the importance of virus-specific immune responses directed against lysosome-targeted autologous SIV sequences for the characterization of the actual breadth and magnitude of cell mediated immunity.

6.5 FUTURE DIRECTIONS

6.5.1 Generation of multispecific T cell responses

Aiming at introducing a multispecific $CD8^+$ and $CD4^+$ T cell responses in HIV-1-infected individuals, we suggest performing additional *in vitro* studies using autologous *gag* mRNA as well as autologous mRNA encoding other important components of the SIV genome such as Env, Pol and Nef and bearing the targeting signal of LAMP-1 . We expect the generation of broad and relevant specific T cell responses.

6.5.2 Investigation of the migratory potential, cytokine profile production, as well as IL-12 production by antigen-transfected DC

To induce optimal T cell responses, DC have to respond to lymph node-derived chemokines. It has been reported that DC electroporated after maturation migrate more efficiently *in vitro* (171, 263) and are more effective in antigen presentation compared to DC electroporated before maturation. We suggest performing similar studies to compare the ability mRNA-nucleofected DC, mock-nucleofected and untreated DC to express CCR7 and to migrate towards its ligand CCL19 (MIP-3β). In addition, we suggest performing similar studies on DC nucleofected with plasmid DNA in order to gain a better understanding on the inefficiency of DNA-transfected DC to stimulate T cells as we observed.

Following exposure to a maturation factor such as LPS or CD40L, DC produce IL-12 for a short period of time and then enter a stage of exhaustion (13). It is critical when using DC-based vaccines to check the ability of DC to secrete cytokines, particularly IL-12. IL-12 is

involved in polarizing the immune response toward a Th1 response *in vivo* and activating naïve $CD8^+$ T cells (225). In addition, IL-12 affects $CD4^+$ T cells to mediate CTL-independent suppression of tumor growth (106) as well as providing $CD4^+$ and $CD8^+$ T cells with a survival signal *in vivo*. There have been reports of reduced IL-12 production in electroporated DC (69, 263). There is still a debate as to which cytokines to add in addition to CD40 ligation leading to optimal IL-12 production (30, 200). This topic requires performing *in vitro* studies using our optimized transfection system.

6.5.3 Testing the immunostimulatory capacity of antigen-transfected DC cotransfected with OX40L and or CD70 in AIDS macaques

We suggest performing more detailed experiments on the effect of coadministration of mRNA encoding costimulatory molecules along with mRNA encoding SIV antigen(s) of choice to DC. We suggest using DC and T cells propagated from macaques during chronic SIV infection with low $CD4^+$ counts, a stage where stimulation of optimal specific T cell responses is needed. Moreover, we suggest using mRNA-transfected DC bearing the lysosomal associated membrane protein (LAMP-1) targeting signal to highlight the importance of these costimulatory molecules towards enhancing Ag-specific $CD8^+$ and promoting $CD4^+$ T cell responses.

6.5.4 *In vivo* studies

The next step in vaccine design following experimental testing is to perform animal studies. We suggest the use of the rhesus macaque model. SIV infection in rhesus monkeys is characterized by a burst of viremia and follows the same course of infection as HIV-1 in humans, regardless of

the route of exposure. Thus, this is an ideal preclinical setting to test the therapeutic potential of DC-based vaccines using virus-derived mRNA.

Current progress has been made in the field of DC-based immunotherapy and HIV-1; however, our work expands on the knowledge in this area by demonstrating the superiority of nucleofection for antigen loading of DC and the ability of mRNA-transfected DC encoding autologous SIV antigen to stimulate strong antigen-specific T cell responses.

BIBLIOGRAPHY

1. Albert ML, Sauter B, Bhardwaj N. Dendritic cells acquire antigen from apoptotic cells and induce class I-restricted CTLs. Nature 1998;392(6671):86-9.

2. Allen TM, Altfeld M, Geer SC, Kalife ET, Moore C, O'Sullivan K M, et al. Selective escape from CD8+ T-cell responses represents a major driving force of human immunodeficiency virus type 1 (HIV-1-1) sequence diversity and reveals constraints on HIV-1-1 evolution. J Virol 2005;79(21):13239-49.

3. Allen TM, Altfeld M, Yu XG, O'Sullivan KM, Lichterfeld M, Le Gall S, et al. Selection, transmission, and reversion of an antigen-processing cytotoxic T-lymphocyte escape mutation in human immunodeficiency virus type 1 infection. J Virol 2004;78(13):7069-78.

4. Allen TM, O'Connor DH, Jing P, Dzuris JL, Mothe BR, Vogel TU, et al. Tat-specific cytotoxic T lymphocytes select for SIV escape variants during resolution of primary viraemia. Nature 2000;407(6802):386-90.

5. Allen TM, Vogel TU, Fuller DH, Mothe BR, Steffen S, Boyson JE, et al. Induction of AIDS virus-specific CTL activity in fresh, unstimulated peripheral blood lymphocytes from rhesus macaques vaccinated with a DNA prime/modified vaccinia virus Ankara boost regimen. Journal of Immunology 2000; 164(9):4968-78.

6. Allen TM, Yu XG, Kalife ET, Reyor LL, Lichterfeld M, John M, et al. De novo generation of escape variant-specific CD8+ T-cell responses following cytotoxic T-lymphocyte escape in chronic human immunodeficiency virus type 1 infection. J Virol 2005; 79(20):12952-60.

7. Altfeld M, Addo MM, Shankarappa R, Lee PK, Allen TM, Yu XG, et al. Enhanced detection of human immunodeficiency virus type 1-specific T-cell responses to highly variable regions by using peptides based on autologous virus sequences. J Virol 2003; 77(13):7330-40.

8. Altfeld M, Rosenberg ES. The role of CD4(+) T helper cells in the cytotoxic T lymphocyte response to HIV-1-1. Curr Opin Immunol 2000; 12(4):375-80.

9. Altfeld M, Rosenberg ES, Shankarappa R, Mukherjee JS, Hecht FM, Eldridge RL, et al. Cellular immune responses and viral diversity in individuals treated during acute and early HIV-1-1 infection. J Exp Med 2001; 193(2):169-80.

10. Amara RR, Villinger F, Altman JD, Lydy SL, O'Neil SP, Staprans SI, et al. Control of a mucosal challenge and prevention of AIDS by a multiprotein DNA/MVA vaccine. Science 2001; 292(5514):69-74.

11. Appay V, Nixon DF, Donahoe SM, Gillespie GM, Dong T, King A, et al. HIV-1-specific CD8(+) T cells produce antiviral cytokines but are impaired in cytolytic function. J Exp Med 2000; 192(1):63-75.

12. Artusio E, Hathaway B, Stanson J, Whiteside TL. Transfection of human monocyte-derived dendritic cells with native tumor DNA induces antigen-specific T-cell responses in vitro. Cancer Biol Ther 2006; 5(12):1624-31.

13. Banchereau J, Briere F, Caux C, Davoust J, Lebecque S, Liu YJ, et al. Immunobiology of dendritic cells. Annu Rev Immunol 2000; 18:767-811.

14. Banchereau J, Paczesny S, Blanco P, Bennett L, Pascual V, Fay J, et al. Dendritic cells: controllers of the immune system and a new promise for immunotherapy. Annals of the New York Academy of Sciences 2003; 987:180-7.

15. Banchereau J, Palucka AK. Dendritic cells as therapeutic vaccines against cancer. Nat Rev Immunol 2005; 5(4):296-306.

16. Banchereau J, Steinman RM. Dendritic cells and the control of immunity. Nature 1998; 392(6673):245-52.

17. Barouch DH, Kunstman J, Glowczwskie J, Kunstman KJ, Egan MA, Peyerl FW, et al. Viral escape from dominant simian immunodeficiency virus epitope-specific cytotoxic T lymphocytes in DNA-vaccinated rhesus monkeys. J Virol 2003; 77(13):7367-75.

18. Barouch DH, Kunstman J, Kuroda MJ, Schmitz JE, Santra S, Peyerl FW, et al. Eventual AIDS vaccine failure in a rhesus monkey by viral escape from cytotoxic T lymphocytes. Nature 2002; 415(6869):335-9.

19. Barouch DH, Pau MG, Custers JH, Koudstaal W, Kostense S, Havenga MJ, et al. Immunogenicity of recombinant adenovirus serotype 35 vaccine in the presence of pre-existing anti-Ad5 immunity. J Immunol 2004; 172(10):6290-7.

20. Barouch DH, Santra S, Schmitz JE, Kuroda MJ, Fu TM, Wagner W, et al. Control of viremia and prevention of clinical AIDS in rhesus monkeys by cytokine-augmented DNA vaccination. Science 2000; 290(5491):486-92.

21. Barratt-Boyes SM, Soloff AC, Gao W, Nwanegbo E, Liu X, Rajakumar PA, et al. Broad cellular immunity with robust memory responses to simian immunodeficiency virus

following serial vaccination with adenovirus 5- and 35-based vectors. J Gen Virol 2006; 87(Pt 1):139-49.

22. Barratt-Boyes SM, Zimmer MI, Harshyne LA, Meyer EM, Watkins SC, Capuano S, 3rd, et al. Maturation and trafficking of monocyte-derived dendritic cells in monkeys: implications for dendritic cell-based vaccines. J Immunol 2000; 164(5):2487-95.

23. Baum C, Forster P, Hegewisch-Becker S, Harbers K. An optimized electroporation protocol applicable to a wide range of cell lines. Biotechniques 1994; 17(6):1058-62.

24. Bertley FM, Kozlowski PA, Wang SW, Chappelle J, Patel J, Sonuyi O, et al. Control of simian/human immunodeficiency virus viremia and disease progression after IL-2-augmented DNA-modified vaccinia virus Ankara nasal vaccination in nonhuman primates. J Immunol 2004; 172(6):3745-57.

25. Betts MR, Nason MC, West SM, De Rosa SC, Migueles SA, Abraham J, et al. HIV-1 nonprogressors preferentially maintain highly functional HIV-1-specific CD8+ T cells. Blood 2006; 107(12):4781-9.

26. Blankson JN, Persaud D, Siliciano RF. The challenge of viral reservoirs in HIV-1-1 infection. Annu Rev Med 2002; 53:557-93.

27. Boczkowski D, Nair SK, Nam JH, Lyerly HK, Gilboa E. Induction of tumor immunity and cytotoxic T lymphocyte responses using dendritic cells transfected with messenger RNA amplified from tumor cells. Cancer Research 2000; 60(4):1028-34.

28. Boczkowski D, Nair SK, Snyder D, Gilboa E. Dendritic cells pulsed with RNA are potent antigen-presenting cells in vitro and in vivo. Journal of Experimental Medicine 1996; 184(2):465-72.

29. Bonini C, Lee SP, Riddell SR, Greenberg PD. Targeting antigen in mature dendritic cells for simultaneous stimulation of CD4+ and CD8+ T cells. J Immunol 2001; 166(8):5250-7.

30. Bontkes HJ, Kramer D, Ruizendaal JJ, Kueter EW, van Tendeloo VF, Meijer CJ, et al. Dendritic cells transfected with interleukin-12 and tumor-associated antigen messenger RNA induce high avidity cytotoxic T cells. Gene Ther 2007; 14(4):366-75.

31. Borrow P, Lewicki H, Hahn BH, Shaw GM, Oldstone MB. Virus-specific CD8+ cytotoxic T-lymphocyte activity associated with control of viremia in primary human immunodeficiency virus type 1 infection. J Virol 1994; 68(9):6103-10.

32. Borrow P, Lewicki H, Wei X, Horwitz MS, Peffer N, Meyers H, et al. Antiviral pressure exerted by HIV-1-1-specific cytotoxic T lymphocytes (CTLs) during primary infection demonstrated by rapid selection of CTL escape virus. Nat Med 1997; 3(2):205-11.

33. Borst J, Hendriks J, Xiao Y. CD27 and CD70 in T cell and B cell activation. Curr Opin Immunol 2005; 17(3):275-81.

34. Brenchley JM, Schacker TW, Ruff LE, Price DA, Taylor JH, Beilman GJ, et al. CD4+ T cell depletion during all stages of HIV-1 disease occurs predominantly in the gastrointestinal tract. J Exp Med 2004; 200(6):749-59.

35. Brown K, Gao W, Alber S, Trichel A, Murphey-Corb M, Watkins SC, et al. Adenovirus-transduced dendritic cells injected into skin or lymph node prime potent simian immunodeficiency virus-specific T cell immunity in monkeys. Journal of Immunology 2003; 171(12):6875-82.

36. Buffa V, Negri DR, Leone P, Borghi M, Bona R, Michelini Z, et al. Evaluation of a self-inactivating lentiviral vector expressing simian immunodeficiency virus gag for induction of specific immune responses in vitro and in vivo. Viral Immunol 2006; 19(4):690-701.

37. Bukczynski J, Wen T, Wang C, Christie N, Routy JP, Boulassel MR, et al. Enhancement of HIV-1-specific CD8 T cell responses by dual costimulation with CD80 and CD137L. J Immunol 2005; 175(10):6378-89.

38. Burton DR, Desrosiers RC, Doms RW, Koff WC, Kwong PD, Moore JP, et al. HIV-1 vaccine design and the neutralizing antibody problem. Nat Immunol 2004;5(3):233-6.

39. Cameron PU, Freudenthal PS, Barker JM, Gezelter S, Inaba K, Steinman RM. Dendritic cells exposed to human immunodeficiency virus type-1 transmit a vigorous cytopathic infection to CD4+ T cells. Science 1992; 257(5068):383-7.

40. Cannon G, Weissman D. RNA based vaccines. DNA Cell Biol 2002; 21(12):953-61.

41. Cannons JL, Lau P, Ghumman B, DeBenedette MA, Yagita H, Okumura K, et al. 4-1BB ligand induces cell division, sustains survival, and enhances effector function of CD4 and CD8 T cells with similar efficacy. J Immunol 2001; 167(3):1313-24.

42. Cao J, McNevin J, Malhotra U, McElrath MJ. Evolution of CD8+ T cell immunity and viral escape following acute HIV-1-1 infection. J Immunol 2003; 171(7):3837-46.

43. Ceppi M, Ruggli N, Tache V, Gerber H, McCullough KC, Summerfield A. Double-stranded secondary structures on mRNA induce type I interferon (IFN alpha/beta) production and maturation of mRNA-transfected monocyte-derived dendritic cells. J Gene Med 2005;7 (4):452-65.

44. Chackerian B, Rudensey LM, Overbaugh J. Specific N-linked and O-linked glycosylation modifications in the envelope V1 domain of simian immunodeficiency virus variants that evolve in the host alter recognition by neutralizing antibodies. J Virol 1997; 71(10):7719-27.

45. Chen AI, McAdam AJ, Buhlmann JE, Scott S, Lupher ML, Jr., Greenfield EA, et al. Ox40-ligand has a critical costimulatory role in dendritic cell:T cell interactions. Immunity 1999; 11(6):689-98.

46. Chen ZW, Craiu A, Shen L, Kuroda MJ, Iroku UC, Watkins DI, et al. Simian immunodeficiency virus evades a dominant epitope-specific cytotoxic T lymphocyte response through a mutation resulting in the accelerated dissociation of viral peptide and MHC class I. Journal of Immunology 2000;164(12):6474-9.

47. Chougnet C, Cohen SS, Kawamura T, Landay AL, Kessler HA, Thomas E, et al. Normal immune function of monocyte-derived dendritic cells from HIV-1-infected individuals: implications for immunotherapy. J Immunol 1999; 163(3):1666-73.

48. Chun TW, Carruth L, Finzi D, Shen X, DiGiuseppe JA, Taylor H, et al. Quantification of latent tissue reservoirs and total body viral load in HIV-1-1 infection. Nature 1997; 387(6629):183-8.

49. Chun TW, Engel D, Berrey MM, Shea T, Corey L, Fauci AS. Early establishment of a pool of latently infected, resting CD4(+) T cells during primary HIV-1-1 infection. Proc Natl Acad Sci U S A 1998; 95(15):8869-73.

50. Chun TW, Fauci AS. Latent reservoirs of HIV-1: obstacles to the eradication of virus. Proc Natl Acad Sci U S A 1999; 96(20):10958-61.

51. Chun TW, Nickle DC, Justement JS, Large D, Semerjian A, Curlin ME, et al. HIV-1-infected individuals receiving effective antiviral therapy for extended periods of time continually replenish their viral reservoir. J Clin Invest 2005; 115(11):3250-5.

52. Cohen J. Public health. AIDS vaccine trial produces disappointment and confusion. Science 2003; 299(5611):1290-1.

53. Condon C, Watkins SC, Celluzzi CM, Thompson K, Falo LD, Jr. DNA-based immunization by in vivo transfection of dendritic cells. Nat Med 1996;2(10):1122-8.

54. Cranmer LD, Trevor KT, Hersh EM. Clinical applications of dendritic cell vaccination in the treatment of cancer. Cancer Immunol Immunother 2004; 53(4):275-306.

55. Croft M. Co-stimulatory members of the TNFR family: keys to effective T-cell immunity? Nat Rev Immunol 2003; 3(8):609-20.

56. Curti A, Pandolfi S, Aluigi M, Isidori A, Alessandrini I, Chiodoni C, et al. Interleukin-12 production by leukemia-derived dendritic cells counteracts the inhibitory effect of leukemic microenvironment on T cells. Exp Hematol 2005; 33(12):1521-30.

57. Daniel MD, Desrosiers RC, Letvin NL, King NW, Schmidt DK, Sehgal P, et al. Simian models for AIDS. Cancer Detect Prev Suppl 1987; 1:501-7.

58. Daniel MD, Kirchhoff F, Czajak SC, Sehgal PK, Desrosiers RC. Protective effects of a live attenuated SIV vaccine with a deletion in the nef gene. Science 1992; 258(5090):1938-41.

59. Dannull J, Nair S, Su Z, Boczkowski D, DeBeck C, Yang B, et al. Enhancing the immunostimulatory function of dendritic cells by transfection with mRNA encoding OX40 ligand. Blood 2005; 105(8):3206-13.

60. Davey RT, Jr., Bhat N, Yoder C, Chun TW, Metcalf JA, Dewar R, et al. HIV-1-1 and T cell dynamics after interruption of highly active antiretroviral therapy (HAART) in patients with a history of sustained viral suppression. Proc Natl Acad Sci U S A 1999;96(26):15109-14.

61. de Arruda LB, Chikhlikar PR, August JT, Marques ET. DNA vaccine encoding human immunodeficiency virus-1 Gag, targeted to the major histocompatibility complex II compartment by lysosomal-associated membrane protein, elicits enhanced long-term memory response. Immunology 2004;112(1):126-33.

62. de Bruyn G, Rossini AJ, Chiu YL, Holman D, Elizaga ML, Frey SE, et al. Safety profile of recombinant canarypox HIV-1 vaccines. Vaccine 2004;22(5-6):704-13.

63. den Haan JM, Bevan MJ. Antigen presentation to CD8+ T cells: cross-priming in infectious diseases. Curr Opin Immunol 2001; 13(4):437-41.

64. Desrosiers RC. Prospects for an AIDS vaccine. Nat Med 2004; 10(3):221-3.

65. Dhodapkar MV, Steinman RM, Krasovsky J, Munz C, Bhardwaj N. Antigen-specific inhibition of effector T cell function in humans after injection of immature dendritic cells. Journal of Experimental Medicine 2001; 193(2):233-8.

66. Doan LX, Li M, Chen C, Yao Q. Virus-like particles as HIV-1-1 vaccines. Rev Med Virol 2005; 15(2):75-88.

67. Dorrell L. Therapeutic immunization for the control of HIV-1-1: where are we now? Int J STD AIDS 2006; 17(7):436-41; quiz 442.

68. Draenert R, Le Gall S, Pfafferott KJ, Leslie AJ, Chetty P, Brander C, et al. Immune selection for altered antigen processing leads to cytotoxic T lymphocyte escape in chronic HIV-1-1 infection. J Exp Med 2004; 199(7):905-15.

69. Dullaers M, Breckpot K, Van Meirvenne S, Bonehill A, Tuyaerts S, Michiels A, et al. Side-by-side comparison of lentivirally transduced and mRNA-electroporated dendritic cells: implications for cancer immunotherapy protocols. Mol Ther 2004; 10(4):768-79.

70. Ellenberger D, Li B, Smith J, Yi H, Folks T, Robinson H, et al. Optimization of a multi-gene HIV-1-1 recombinant subtype CRF02_AG DNA vaccine for expression of multiple immunogenic forms. Virology 2004; 319(1):118-30.

71. Engelmayer J, Larsson M, Lee A, Lee M, Cox WI, Steinman RM, et al. Mature dendritic cells infected with canarypox virus elicit strong anti-human immunodeficiency virus CD8+ and CD4+ T-cell responses from chronically infected individuals. J Virol 2001; 75(5):2142-53.

72. Engelmayer J, Larsson M, Subklewe M, Chahroudi A, Cox WI, Steinman RM, et al. Vaccinia virus inhibits the maturation of human dendritic cells: a novel mechanism of immune evasion. J Immunol 1999;163(12):6762-8.

73. Evans DT, O'Connor DH, Jing P, Dzuris JL, Sidney J, da Silva J, et al. Virus-specific cytotoxic T-lymphocyte responses select for amino-acid variation in simian immunodeficiency virus Env and Nef. Nature Medicine 1999;5(11):1270-6.

74. Fauci AS. Host factors and the pathogenesis of HIV-1-induced disease. Nature 1996; 384(6609):529-34.

75. Finzi D, Hermankova M, Pierson T, Carruth LM, Buck C, Chaisson RE, et al. Identification of a reservoir for HIV-1-1 in patients on highly active antiretroviral therapy. Science 1997; 278(5341):1295-300.

76. Frahm N, Kiepiela P, Adams S, Linde CH, Hewitt HS, Sango K, et al. Control of human immunodeficiency virus replication by cytotoxic T lymphocytes targeting subdominant epitopes. Nat Immunol 2006; 7(2):173-8.

77. Frankel SS, Tenner-Racz K, Racz P, Wenig BM, Hansen CH, Heffner D, et al. Active replication of HIV-1-1 at the lymphoepithelial surface of the tonsil. Am J Pathol 1997; 151(1):89-96.

78. Frankel SS, Wenig BM, Burke AP, Mannan P, Thompson LD, Abbondanzo SL, et al. Replication of HIV-1-1 in dendritic cell-derived syncytia at the mucosal surface of the adenoid. Science 1996; 272(5258):115-7.

79. Frasca L, Piazza C, Piccolella E. CD4+ T cells orchestrate both amplification and deletion of CD8+ T cells. Crit Rev Immunol 1998;18(6):569-94.

80. Friedrich TC, Dodds EJ, Yant LJ, Vojnov L, Rudersdorf R, Cullen C, et al. Reversion of CTL escape-variant immunodeficiency viruses in vivo. Nat Med 2004;10(3):275-81.

81. Garcia F, Lejeune M, Climent N, Gil C, Alcami J, Morente V, et al. Therapeutic immunization with dendritic cells loaded with heat-inactivated autologous HIV-1-1 in patients with chronic HIV-1-1 infection. J Infect Dis 2005; 191(10):1680-5.

82. Gartner A, Collin L, Lalli G. Nucleofection of primary neurons. Methods Enzymol 2006; 406:374-88.

83. Gaschen B, Taylor J, Yusim K, Foley B, Gao F, Lang D, et al. Diversity considerations in HIV-1-1 vaccine selection. Science 2002;296(5577):2354-60.

84. Geijtenbeek TB, Kwon DS, Torensma R, van Vliet SJ, van Duijnhoven GC, Middel J, et al. DC-SIGN, a dendritic cell-specific HIV-1-1-binding protein that enhances trans-infection of T cells. Cell 2000;100(5):587-97.

85. Gilboa E. The promise of cancer vaccines. Nature Reviews Cancer 2004;4(5):401-11.

86. Gilboa E. DC-based cancer vaccines. J Clin Invest 2007; 117(5):1195-203.

87. Glass JD, Johnson RT. Human immunodeficiency virus and the brain. Annu Rev Neurosci 1996; 19:1-26.

88. Goulder PJ, Brander C, Tang Y, Tremblay C, Colbert RA, Addo MM, et al. Evolution and transmission of stable CTL escape mutations in HIV-1 infection. Nature 2001; 412(6844):334-8.

89. Goulder PJ, Phillips RE, Colbert RA, McAdam S, Ogg G, Nowak MA, et al. Late escape from an immunodominant cytotoxic T-lymphocyte response associated with progression to AIDS. Nat Med 1997; 3(2):212-7.

90. Goulder PJ, Rowland-Jones SL, McMichael AJ, Walker BD. Anti-HIV-1 cellular immunity: recent advances towards vaccine design. AIDS 1999; 13 Suppl A:S121-36.

91. Goulder PJ, Walker BD. The great escape - AIDS viruses and immune control. Nat Med 1999; 5(11):1233-5.

92. Goulder PJ, Watkins DI. HIV-1 and SIV CTL escape: implications for vaccine design. Nat Rev Immunol 2004; 4(8):630-40.

93. Gramaglia I, Jember A, Pippig SD, Weinberg AD, Killen N, Croft M. The OX40 costimulatory receptor determines the development of CD4 memory by regulating primary clonal expoansion. J Immunol 2000; 165(6):3043-50.

94. Granelli-Piperno A, Zhong L, Haslett P, Jacobson J, Steinman RM. Dendritic cells, infected with vesicular stomatitis virus-pseudotyped HIV-1-1, present viral antigens to CD4+ and CD8+ T cells from HIV-1-1-infected individuals. J Immunol 2000; 165(11):6620-6.

95. Gresch O, Engel FB, Nesic D, Tran TT, England HM, Hickman ES, et al. New non-viral method for gene transfer into primary cells. Methods 2004; 33(2):151-63.

96. Grunebach F, Muller MR, Brossart P. New developments in dendritic cell-based vaccinations: RNA translated into clinics. Cancer Immunol Immunother 2005; 54(6):517-25.

97. Grunebach F, Muller MR, Brossart P. RNA transfection of dendritic cells. Methods Mol Med 2005; 109:47-54.

98. Grunebach F, Muller MR, Nencioni A, Brossart P. Delivery of tumor-derived RNA for the induction of cytotoxic T-lymphocytes. Gene Ther 2003; 10(5):367-74.

99. Guermonprez P, Saveanu L, Kleijmeer M, Davoust J, Van Endert P, Amigorena S. ER-phagosome fusion defines an MHC class I cross-presentation compartment in dendritic cells. Nature 2003;425(6956):397-402.

100. Harari A, Rizzardi GP, Ellefsen K, Ciuffreda D, Champagne P, Bart PA, et al. Analysis of HIV-1-1- and CMV-specific memory CD4 T-cell responses during primary and chronic infection. Blood 2002; 100(4):1381-7.

101. Heath WR, Carbone FR. Cross-presentation in viral immunity and self-tolerance. Nat Rev Immunol 2001; 1(2):126-34.

102. Heine A, Grunebach F, Holderried T, Appel S, Weck MM, Dorfel D, et al. Transfection of dendritic cells with in vitro-transcribed CMV RNA induces polyclonal CD8+- and CD4+-mediated CMV-specific T cell responses. Mol Ther 2006; 13(2):280-8.

103. Heiser A, Coleman D, Dannull J, Yancey D, Maurice MA, Lallas CD, et al. Autologous dendritic cells transfected with prostate-specific antigen RNA stimulate CTL responses against metastatic prostate tumors. J Clin Invest 2002; 109(3):409-17.

104. Heiser A, Dahm P, Yancey DR, Maurice MA, Boczkowski D, Nair SK, et al. Human dendritic cells transfected with RNA encoding prostate-specific antigen stimulate prostate-specific CTL responses in vitro. Journal of Immunology 2000; 164(10):5508-14.

105. Hermankova M, Siliciano JD, Zhou Y, Monie D, Chadwick K, Margolick JB, et al. Analysis of human immunodeficiency virus type 1 gene expression in latently infected resting CD4+ T lymphocytes in vivo. J Virol 2003; 77(13):7383-92.

106. Hess SD, Egilmez NK, Bailey N, Anderson TM, Mathiowitz E, Bernstein SH, et al. Human CD4+ T cells present within the microenvironment of human lung tumors are mobilized by the local and sustained release of IL-12 to kill tumors in situ by indirect effects of IFN-gamma. J Immunol 2003; 170(1):400-12.

107. Hirsch VM, Dapolito G, Goeken R, Campbell BJ. Phylogeny and natural history of the primate lentiviruses, SIV and HIV-1. Curr Opin Genet Dev 1995; 5(6):798-806.

108. Hirsch VM, Fuerst TR, Sutter G, Carroll MW, Yang LC, Goldstein S, et al. Patterns of viral replication correlate with outcome in simian immunodeficiency virus (SIV)-infected macaques: effect of prior immunization with a trivalent SIV vaccine in modified vaccinia virus Ankara. J Virol 1996; 70(6):3741-52.

109. Hirsch VM, Olmsted RA, Murphey-Corb M, Purcell RH, Johnson PR. An African primate lentivirus (SIVsm) closely related to HIV-1-2. Nature 1989;339(6223):389-92.

110. Hogg RS, Heath KV, Yip B, Craib KJ, O'Shaughnessy MV, Schechter MT, et al. Improved survival among HIV-1-infected individuals following initiation of antiretroviral therapy. JAMA 1998; 279(6):450-4.

111. Hou WS, Van Parijs L. A Bcl-2-dependent molecular timer regulates the lifespan and immunogenicity of dendritic cells. Nat Immunol 2004; 5(6):583-9.

112. Hu J, Gardner MB, Miller CJ. Simian immunodeficiency virus rapidly penetrates the cervicovaginal mucosa after intravaginal inoculation and infects intraepithelial dendritic cells. J Virol 2000; 74(13):6087-95.

113. Hu J, Miller CJ, O'Doherty U, Marx PA, Pope M. The dendritic cell-T cell milieu of the lymphoid tissue of the tonsil provides a locale in which SIV can reside and propagate at chronic stages of infection. AIDS Res Hum Retroviruses 1999; 15(14):1305-14.

114. Huang XL, Fan Z, Zheng L, Borowski L, Li H, Thomas EK, et al. Priming of human immunodeficiency virus type 1 (HIV-1-1)-specific CD8+ T cell responses by dendritic cells loaded with HIV-1-1 proteins. J Infect Dis 2003; 187(2):315-9.

115. Inaba K, Turley S, Iyoda T, Yamaide F, Shimoyama S, Reis e Sousa C, et al. The formation of immunogenic major histocompatibility complex class II-peptide ligands in lysosomal compartments of dendritic cells is regulated by inflammatory stimuli. J Exp Med 2000; 191(6):927-36.

116. Jacobsen F, Mertens-Rill J, Beller J, Hirsch T, Daigeler A, Langer S, et al. Nucleofection: a new method for cutaneous gene transfer? J Biomed Biotechnol 2006;2006(5):26060.

117. Janssen EM, Lemmens EE, Wolfe T, Christen U, von Herrath MG, Schoenberger SP. CD4+ T cells are required for secondary expansion and memory in CD8+ T lymphocytes. Nature 2003; 421(6925):852-6.

118. Jenne L, Schuler G, Steinkasserer A. Viral vectors for dendritic cell-based immunotherapy. Trends Immunol 2001; 22(2):102-7.

119. Ji H, Wang TL, Chen CH, Pai SI, Hung CF, Lin KY, et al. Targeting human papillomavirus type 16 E7 to the endosomal/lysosomal compartment enhances the antitumor immunity of DNA vaccines against murine human papillomavirus type 16 E7-expressing tumors. Hum Gene Ther 1999; 10(17):2727-40.

120. Jin X, Bauer DE, Tuttleton SE, Lewin S, Gettie A, Blanchard J, et al. Dramatic rise in plasma viremia after CD8(+) T cell depletion in simian immunodeficiency virus-infected macaques. J Exp Med 1999; 189(6):991-8.

121. Johnston MI, Fauci AS. An HIV-1 vaccine--evolving concepts. N Engl J Med 2007; 356(20):2073-81.

122. Jones NA, Wei X, Flower DR, Wong M, Michor F, Saag MS, et al. Determinants of human immunodeficiency virus type 1 escape from the primary CD8+ cytotoxic T lymphocyte response. J Exp Med 2004; 200(10):1243-56.

123. Kalady MF, Onaitis MW, Padilla KM, Emani S, Tyler DS, Pruitt SK. Enhanced dendritic cell antigen presentation in RNA-based immunotherapy. J Surg Res 2002; 105(1):17-24.

124. Kalams SA, Buchbinder SP, Rosenberg ES, Billingsley JM, Colbert DS, Jones NG, et al. Association between virus-specific cytotoxic T-lymphocyte and helper responses in human immunodeficiency virus type 1 infection. J Virol 1999; 73(8):6715-20.

125. Kalams SA, Walker BD. The critical need for CD4 help in maintaining effective cytotoxic T lymphocyte responses. J Exp Med 1998;188(12):2199-204.

126. Kalinski P, Hilkens CM, Wierenga EA, Kapsenberg ML. T-cell priming by type-1 and type-2 polarized dendritic cells: the concept of a third signal. Immunol Today 1999; 20(12):561-7.

127. Kanki PJ, McLane MF, King NW, Jr., Letvin NL, Hunt RD, Sehgal P, et al. Serologic identification and characterization of a macaque T-lymphotropic retrovirus closely related to HTLV-III. Science 1985; 228(4704):1199-201.

128. Kariko K, Ni H, Capodici J, Lamphier M, Weissman D. mRNA is an endogenous ligand for Toll-like receptor 3. J Biol Chem 2004; 279(13):12542-50.

129. Kavanagh DG, Kaufmann DE, Sunderji S, Frahm N, Le Gall S, Boczkowski D, et al. Expansion of HIV-1-specific CD4+ and CD8+ T cells by dendritic cells transfected with mRNA encoding cytoplasm- or lysosome-targeted Nef. Blood 2006; 107(5):1963-9.

130. Kedl RM, Schaefer BC, Kappler JW, Marrack P. T cells down-modulate peptide-MHC complexes on APCs in vivo. Nat Immunol 2002;3(1):27-32.

131. Kelleher AD, Long C, Holmes EC, Allen RL, Wilson J, Conlon C, et al. Clustered mutations in HIV-1-1 gag are consistently required for escape from HLA-B27-restricted cytotoxic T lymphocyte responses. J Exp Med 2001; 193(3):375-86.

132. Kestler HW, 3rd, Mori K, Silva DP, Kodama T, King NW, Daniel MD, et al. Nef genes of SIV. J Med Primatol 1990; 19(3-4):421-9.

133. Kijak GH, McCutchan FE. HIV-1 diversity, molecular epidemiology, and the role of recombination. Curr Infect Dis Rep 2005; 7(6):480-8.

134. Klenerman P, Lechner F, Kantzanou M, Ciurea A, Hengartner H, Zinkernagel R. Viral escape and the failure of cellular immune responses. Science 2000; 289(5487):2003.

135. Korber B, Gaschen B, Yusim K, Thakallapally R, Kesmir C, Detours V. Evolutionary and immunological implications of contemporary HIV-1-1 variation. Br Med Bull 2001; 58:19-42.

136. Koup RA, Safrit JT, Cao Y, Andrews CA, McLeod G, Borkowsky W, et al. Temporal association of cellular immune responses with the initial control of viremia in primary human immunodeficiency virus type 1 syndrome. J Virol 1994; 68(7):4650-5.

137. Kuroda MJ, Schmitz JE, Charini WA, Nickerson CE, Lifton MA, Lord CI, et al. Emergence of CTL coincides with clearance of virus during primary simian immunodeficiency virus infection in rhesus monkeys. J Immunol 1999; 162(9):5127-33.

138. Kwon DS, Gregorio G, Bitton N, Hendrickson WA, Littman DR. DC-SIGN-mediated internalization of HIV-1 is required for trans-enhancement of T cell infection. Immunity 2002; 16(1):135-44.

139. Kyte JA, Kvalheim G, Aamdal S, Saeboe-Larssen S, Gaudernack G. Preclinical full-scale evaluation of dendritic cells transfected with autologous tumor-mRNA for melanoma vaccination. Cancer Gene Ther 2005; 12(6):579-91.

140. Landi A, Babiuk LA, van Drunen Littel-van den Hurk S. High transfection efficiency, gene expression, and viability of monocyte-derived human dendritic cells after nonviral gene transfer. J Leukoc Biol 2007.

141. Lanzavecchia A. Mechanisms of antigen uptake for presentation. Curr Opin Immunol 1996; 8(3):348-54.

142. Larregina AT, Morelli AE, Tkacheva O, Erdos G, Donahue C, Watkins SC, et al. Highly efficient expression of transgenic proteins by naked DNA-transfected dendritic cells through terminal differentiation. Blood 2004; 103(3):811-9.

143. Learmont JC, Geczy AF, Mills J, Ashton LJ, Raynes-Greenow CH, Garsia RJ, et al. Immunologic and virologic status after 14 to 18 years of infection with an attenuated strain of HIV-1-1. A report from the Sydney Blood Bank Cohort. N Engl J Med 1999; 340(22):1715-22.

144. Lee JK, Stewart-Jones G, Dong T, Harlos K, Di Gleria K, Dorrell L, et al. T cell cross-reactivity and conformational changes during TCR engagement. J Exp Med 2004; 200(11):1455-66.

145. Lee SK, Xu Z, Lieberman J, Shankar P. The functional CD8 T cell response to HIV-1 becomes type-specific in progressive disease. J Clin Invest 2002;110(9):1339-47.

146. Lenz P, Bacot SM, Frazier-Jessen MR, Feldman GM. Nucleoporation of dendritic cells: efficient gene transfer by electroporation into human monocyte-derived dendritic cells. FEBS Lett 2003; 538(1-3):149-54.

147. Leslie AJ, Pfafferott KJ, Chetty P, Draenert R, Addo MM, Feeney M, et al. HIV-1 evolution: CTL escape mutation and reversion after transmission. Nat Med 2004; 10(3):282-9.

148. Letvin NL. Progress toward an HIV-1 vaccine. Annu Rev Med 2005;56:213-23.

149. Letvin NL. Progress and obstacles in the development of an AIDS vaccine. Nat Rev Immunol 2006; 6(12):930-9.

150. Letvin NL, King NW. Immunologic and pathologic manifestations of the infection of rhesus monkeys with simian immunodeficiency virus of macaques. J Acquir Immune Defic Syndr 1990; 3(11):1023-40.

151. Li Q, Duan L, Estes JD, Ma ZM, Rourke T, Wang Y, et al. Peak SIV replication in resting memory CD4+ T cells depletes gut lamina propria CD4+ T cells. Nature 2005;434(7037):1148-52.

152. Liao X, Li Y, Bonini C, Nair S, Gilboa E, Greenberg PD, et al. Transfection of RNA encoding tumor antigens following maturation of dendritic cells leads to prolonged presentation of antigen and the generation of high-affinity tumor-reactive cytotoxic T lymphocytes. Molecular Therapy: the Journal of the American Society of Gene Therapy 2004; 9(5):757-64.

153. Lichterfeld M, Kaufmann DE, Yu XG, Mui SK, Addo MM, Johnston MN, et al. Loss of HIV-1-1-specific CD8+ T cell proliferation after acute HIV-1-1 infection and restoration by vaccine-induced HIV-1-1-specific CD4+ T cells. J Exp Med 2004; 200(6):701-12.

154. Lifson JD, Nowak MA, Goldstein S, Rossio JL, Kinter A, Vasquez G, et al. The extent of early viral replication is a critical determinant of the natural history of simian immunodeficiency virus infection. J Virol 1997; 71(12):9508-14.

155. Lisziewicz J, Gabrilovich DI, Varga G, Xu J, Greenberg PD, Arya SK, et al. Induction of potent human immunodeficiency virus type 1-specific T-cell-restricted immunity by genetically modified dendritic cells. J Virol 2001; 75(16):7621-8.

156. Lu W, Andrieu JM. In vitro human immunodeficiency virus eradication by autologous CD8(+) T cells expanded with inactivated-virus-pulsed dendritic cells. Journal of Virology 2001; 75(19):8949-56.

157. Lu W, Arraes LC, Ferreira WT, Andrieu J-M. Therapeutic dendritic-cell vaccine for chronic HIV-1-1 infection. Nature Medicine 2004;10(12):1359-65.

158. Lu W, Wu X, Lu Y, Guo W, Andrieu JM. Therapeutic dendritic-cell vaccine for simian AIDS. Nat Med 2003;9(1):27-32.

159. Lu Y, Raviprakash K, Leao IC, Chikhlikar PR, Ewing D, Anwar A, et al. Dengue 2 PreM-E/LAMP chimera targeted to the MHC class II compartment elicits long-lasting neutralizing antibodies. Vaccine 2003; 21(17-18):2178-89.

160. Lundqvist A, Noffz G, Pavlenko M, Saeboe-Larssen S, Fong T, Maitland N, et al. Nonviral and viral gene transfer into different subsets of human dendritic cells yield comparable efficiency of transfection. J Immunother (1997) 2002; 25(6):445-54.

161. Luo D, Saltzman WM. Synthetic DNA delivery systems. Nat Biotechnol 2000; 18(1):33-7.

162. Maranon C, Desoutter JF, Hoeffel G, Cohen W, Hanau D, Hosmalin A. Dendritic cells cross-present HIV-1 antigens from live as well as apoptotic infected CD4+ T lymphocytes. Proc Natl Acad Sci U S A 2004; 101(16):6092-7.

163. Marques ET, Jr., Chikhlikar P, de Arruda LB, Leao IC, Lu Y, Wong J, et al. HIV-1-1 p55Gag encoded in the lysosome-associated membrane protein-1 as a DNA plasmid vaccine chimera is highly expressed, traffics to the major histocompatibility class II compartment, and elicits enhanced immune responses. J Biol Chem 2003;278(39):37926-36.

164. MartIn-Fontecha A, Sebastiani S, Hopken UE, Uguccioni M, Lipp M, Lanzavecchia A, et al. Regulation of dendritic cell migration to the draining lymph node: impact on T lymphocyte traffic and priming. J Exp Med 2003; 198(4):615-21.

165. Mattapallil JJ, Douek DC, Hill B, Nishimura Y, Martin M, Roederer M. Massive infection and loss of memory CD4+ T cells in multiple tissues during acute SIV infection. Nature 2005; 434(7037):1093-7.

166. McDonald D, Wu L, Bohks SM, KewalRamani VN, Unutmaz D, Hope TJ. Recruitment of HIV-1 and its receptors to dendritic cell-T cell junctions. Science 2003; 300(5623):1295-7.

167. McMichael AJ. HIV-1 vaccines. Annu Rev Immunol 2006;24:227-55.

168. Mehandru S, Poles MA, Tenner-Racz K, Horowitz A, Hurley A, Hogan C, et al. Primary HIV-1-1 infection is associated with preferential depletion of CD4+ T lymphocytes from effector sites in the gastrointestinal tract. J Exp Med 2004;200(6):761-70.

169. Melhem NM, Liu XD, Boczkowski D, Gilboa E, Barratt-Boyes SM. Robust CD4(+) and CD8(+) T cell responses to SIV using mRNA-transfected DC expressing autologous viral Ag. Eur J Immunol 2007.

170. Mellors JW, Kingsley LA, Rinaldo CR, Jr., Todd JA, Hoo BS, Kokka RP, et al. Quantitation of HIV-1-1 RNA in plasma predicts outcome after seroconversion. Ann Intern Med 1995; 122(8):573-9.

171. Michiels A, Tuyaerts S, Bonehill A, Corthals J, Breckpot K, Heirman C, et al. Electroporation of immature and mature dendritic cells: implications for dendritic cell-based vaccines. Gene Ther 2005; 12(9):772-82.

172. Migueles SA, Laborico AC, Shupert WL, Sabbaghian MS, Rabin R, Hallahan CW, et al. HIV-1-specific CD8+ T cell proliferation is coupled to perforin expression and is maintained in nonprogressors. Nat Immunol 2002; 3(11):1061-8.

173. Miller MD, Yamamoto H, Hughes AL, Watkins DI, Letvin NL. Definition of an epitope and MHC class I molecule recognized by gag-specific cytotoxic T lymphocytes in SIVmac-infected rhesus monkeys. J Immunol 1991; 147(1):320-9.

174. Mitchell DA, Nair SK. RNA transfected dendritic cells as cancer vaccines. J Clin Invest 2000; 106(9): 1065-9.

175. Moore CB, John M, James IR, Christiansen FT, Witt CS, Mallal SA. Evidence of HIV-1-1 adaptation to HLA-restricted immune responses at a population level. Science 2002; 296(5572):1439-43.

176. Morelli AE, Larregina AT, Ganster RW, Zahorchak AF, Plowey JM, Takayama T, et al. Recombinant adenovirus induces maturation of dendritic cells via an NF-kappaB-dependent pathway. J Virol 2000; 74(20):9617-28.

177. Morse MA, Coleman RE, Akabani G, Niehaus N, Coleman D, Lyerly HK. Migration of human dendritic cells after injection in patients with metastatic malignancies. Cancer Res 1999; 59(1):56-8.

178. Morse MA, Lyerly HK, Gilboa E, Thomas E, Nair SK. Optimization of the sequence of antigen loading and CD40-ligand-induced maturation of dendritic cells. Cancer Research 1998; 58(14):2965-8.

179. Morse MA, Nair SK, Mosca PJ, Hobeika AC, Clay TM, Deng Y, et al. Immunotherapy with autologous, human dendritic cells transfected with carcinoembryonic antigen mRNA. Cancer Investigation 2003; 21(3):341-9.

180. Murphey-Corb M, Martin LN, Davison-Fairburn B, Montelaro RC, Miller M, West M, et al. A formalin-inactivated whole SIV vaccine confers protection in macaques. Science 1989; 246(4935):1293-7.

181. Mwau M, Cebere I, Sutton J, Chikoti P, Winstone N, Wee EG, et al. A human immunodeficiency virus 1 (HIV-1-1) clade A vaccine in clinical trials: stimulation of HIV-1-specific T-cell responses by DNA and recombinant modified vaccinia virus Ankara (MVA) vaccines in humans. J Gen Virol 2004;85(Pt 4):911-9.

182. Nagaraj S, Ziske C, Schmidt-Wolf IG. Human cytokine-induced killer cells have enhanced in vitro cytolytic activity via non-viral interleukin-2 gene transfer. Genet Vaccines Ther 2004; 2(1):12.

183. Nair S, Boczkowski D, Moeller B, Dewhirst M, Vieweg J, Gilboa E. Synergy between tumor immunotherapy and antiangiogenic therapy. Blood 2003;102(3):964-71.

184. Nair S, McLaughlin C, Weizer A, Su Z, Boczkowski D, Dannull J, et al. Injection of immature dendritic cells into adjuvant-treated skin obviates the need for ex vivo maturation. J Immunol 2003; 171(11):6275-82.

185. Nair SK, Boczkowski D, Morse M, Cumming RI, Lyerly HK, Gilboa E. Induction of primary carcinoembryonic antigen (CEA)-specific cytotoxic T lymphocytes in vitro using human dendritic cells transfected with RNA. Nature Biotechnology 1998; 16(4):364-9.

186. Nair SK, Morse M, Boczkowski D, Cumming RI, Vasovic L, Gilboa E, et al. Induction of tumor-specific cytotoxic T lymphocytes in cancer patients by autologous tumor RNA-transfected dendritic cells. Annals of Surgery 2002; 235(4):540-9.

187. Napolitani G, Rinaldi A, Bertoni F, Sallusto F, Lanzavecchia A. Selected Toll-like receptor agonist combinations synergistically trigger a T helper type 1-polarizing program in dendritic cells. Nat Immunol 2005; 6(8):769-76.

188. Nehete PN, Chitta S, Hossain MM, Hill L, Bernacky BJ, Baze W, et al. Protection against chronic infection and AIDS by an HIV-1 envelope peptide-cocktail vaccine in a pathogenic SHIV-1-rhesus model. Vaccine 2001; 20(5-6):813-25.

189. Nehete PN, Gambhira R, Nehete BP, Sastry KJ. Dendritic cells enhance detection of antigen-specific cellular immune responses by lymphocytes from rhesus macaques immunized with an HIV-1 envelope peptide cocktail vaccine. J Med Primatol 2003; 32(2):67-73.

190. Nehete PN, Nehete BP, Manuri P, Hill L, Palmer JL, Sastry KJ. Protection by dendritic cells-based HIV-1 synthetic peptide cocktail vaccine: preclinical studies in the SHIV-1-rhesus model. Vaccine 2005;23(17-18):2154-9.

191. Nestle FO, Farkas A, Conrad C. Dendritic-cell-based therapeutic vaccination against cancer. Curr Opin Immunol 2005;17(2):163-9.

192. Netea MG, van der Graaf C, Van der Meer JW, Kullberg BJ. Toll-like receptors and the host defense against microbial pathogens: bringing specificity to the innate-immune system. J Leukoc Biol 2004; 75(5):749-55.

193. Newton PJ, Weller IV, Williams IG, Miller RF, Copas A, Tedder RS, et al. Monocyte derived dendritic cells from HIV-1-1 infected individuals partially reconstitute CD4 T-cell responses. AIDS 2006; 20(2):171-80.

194. Norris PJ, Sumaroka M, Brander C, Moffett HF, Boswell SL, Nguyen T, et al. Multiple effector functions mediated by human immunodeficiency virus-specific CD4(+) T-cell clones. J Virol 2001; 75(20):9771-9.

195. Novitsky V, Rybak N, McLane MF, Gilbert P, Chigwedere P, Klein I, et al. Identification of human immunodeficiency virus type 1 subtype C Gag-, Tat-, Rev-, and Nef-specific elispot-based cytotoxic T-lymphocyte responses for AIDS vaccine design. J Virol 2001; 75(19):9210-28.

196. Novitsky V, Smith UR, Gilbert P, McLane MF, Chigwedere P, Williamson C, et al. Human immunodeficiency virus type 1 subtype C molecular phylogeny: consensus sequence for an AIDS vaccine design? J Virol 2002; 76(11):5435-51.

197. Ochsenbein AF, Riddell SR, Brown M, Corey L, Baerlocher GM, Lansdorp PM, et al. CD27 expression promotes long-term survival of functional effector-memory CD8+ cytotoxic T lymphocytes in HIV-1-infected patients. J Exp Med 2004; 200(11):1407-17.

198. O'Connor DH, Allen TM, Vogel TU, Jing P, DeSouza IP, Dodds E, et al. Acute phase cytotoxic T lymphocyte escape is a hallmark of simian immunodeficiency virus infection. Nature Medicine 2002; 8(5):493-9.

199. Ogg GS, Jin X, Bonhoeffer S, Dunbar PR, Nowak MA, Monard S, et al. Quantitation of HIV-1-1-specific cytotoxic T lymphocytes and plasma load of viral RNA. Science 1998; 279(5359):2103-6.

200. Onaitis MW, Kalady MF, Emani S, Abdel-Wahab Z, Tyler DS, Pruitt SK. CD40 ligand is essential for generation of specific cytotoxic T cell responses in RNA-pulsed dendritic cell immunotherapy. Surgery 2003; 134(2):300-5.

201. Oshima H, Nakano H, Nohara C, Kobata T, Nakajima A, Jenkins NA, et al. Characterization of murine CD70 by molecular cloning and mAb. Int Immunol 1998; 10(4):517-26.

202. Oxenius A, Price DA, Gunthard HF, Dawson SJ, Fagard C, Perrin L, et al. Stimulation of HIV-1-specific cellular immunity by structured treatment interruption fails to enhance viral control in chronic HIV-1 infection. Proc Natl Acad Sci U S A 2002;99(21):13747-52.

203. Palella FJ, Jr., Delaney KM, Moorman AC, Loveless MO, Fuhrer J, Satten GA, et al. Declining morbidity and mortality among patients with advanced human immunodeficiency virus infection. HIV-1 Outpatient Study Investigators. N Engl J Med 1998; 338(13):853-60.

204. Palmowski MJ, Choi EM, Hermans IF, Gilbert SC, Chen JL, Gileadi U, et al. Competition between CTL narrows the immune response induced by prime-boost vaccination protocols. J Immunol 2002; 168(9):4391-8.

205. Pantaleo G, Koup RA. Correlates of immune protection in HIV-1-1 infection: what we know, what we don't know, what we should know. Nat Med 2004; 10(8):806-10.

206. Peyerl FW, Barouch DH, Letvin NL. Structural constraints on viral escape from HIV-1- and SIV-specific cytotoxic T-lymphocytes. Viral Immunol 2004;17(2):144-51.

207. Peyerl FW, Barouch DH, Yeh WW, Bazick HS, Kunstman J, Kunstman KJ, et al. Simian-human immunodeficiency virus escape from cytotoxic T-lymphocyte recognition at a structurally constrained epitope. J Virol 2003; 77(23):12572-8.

208. Piacenti FJ. An update and review of antiretroviral therapy. Pharmacotherapy 2006; 26(8):1111-33.

209. Picker LJ, Hagen SI, Lum R, Reed-Inderbitzin EF, Daly LM, Sylwester AW, et al. Insufficient production and tissue delivery of CD4+ memory T cells in rapidly progressive simian immunodeficiency virus infection. J Exp Med 2004; 200(10):1299-314.

210. Pierson T, McArthur J, Siliciano RF. Reservoirs for HIV-1-1: mechanisms for viral persistence in the presence of antiviral immune responses and antiretroviral therapy. Annu Rev Immunol 2000; 18:665-708.

211. Ponsaerts P, Van den Bosch G, Cools N, Van Driessche A, Nijs G, Lenjou M, et al. Messenger RNA electroporation of human monocytes, followed by rapid in vitro differentiation, leads to highly stimulatory antigen-loaded mature dendritic cells. Journal of Immunology 2002; 169(4):1669-75.

212. Ponsaerts P, Van Tendeloo VF, Berneman ZN. Cancer immunotherapy using RNA-loaded dendritic cells. Clin Exp Immunol 2003; 134(3):378-84.

213. Pope M, Betjes MG, Romani N, Hirmand H, Cameron PU, Hoffman L, et al. Conjugates of dendritic cells and memory T lymphocytes from skin facilitate productive infection with HIV-1-1. Cell 1994; 78(3):389-98.

214. Pope M, Elmore D, Ho D, Marx P. Dendrite cell-T cell mixtures, isolated from the skin and mucosae of macaques, support the replication of SIV. AIDS Res Hum Retroviruses 1997; 13(10):819-27.

215. Pope M, Gezelter S, Gallo N, Hoffman L, Steinman RM. Low levels of HIV-1-1 infection in cutaneous dendritic cells promote extensive viral replication upon binding to memory CD4+ T cells. J Exp Med 1995; 182(6):2045-56.

216. Rambaut A, Posada D, Crandall KA, Holmes EC. The causes and consequences of HIV-1 evolution. Nat Rev Genet 2004;5(1):52-61.

217. Raviprakash K, Marques E, Ewing D, Lu Y, Phillips I, Porter KR, et al. Synergistic neutralizing antibody response to a dengue virus type 2 DNA vaccine by incorporation of lysosome-associated membrane protein sequences and use of plasmid expressing GM-CSF. Virology 2001; 290(1):74-82.

218. Richman DD, Wrin T, Little SJ, Petropoulos CJ. Rapid evolution of the neutralizing antibody response to HIV-1 type 1 infection. Proc Natl Acad Sci U S A 2003; 100(7):4144-9.

219. Ridge JP, Di Rosa F, Matzinger P. A conditioned dendritic cell can be a temporal bridge between a CD4+ T-helper and a T-killer cell. Nature 1998;393(6684):474-8.

220. Rinaldo C, Huang XL, Fan ZF, Ding M, Beltz L, Logar A, et al. High levels of anti-human immunodeficiency virus type 1 (HIV-1-1) memory cytotoxic T-lymphocyte activity and low viral load are associated with lack of disease in HIV-1-1-infected long-term nonprogressors. J Virol 1995; 69(9):5838-42.

221. Rinaldo CR, Jr., Piazza P. Virus infection of dendritic cells: portal for host invasion and host defense. Trends Microbiol 2004; 12(7):337-45.

222. Rose NF, Marx PA, Luckay A, Nixon DF, Moretto WJ, Donahoe SM, et al. An effective AIDS vaccine based on live attenuated vesicular stomatitis virus recombinants. Cell 2001; 106(5):539-49.

223. Rosenberg ES, Billingsley JM, Caliendo AM, Boswell SL, Sax PE, Kalams SA, et al. Vigorous HIV-1-1-specific CD4+ T cell responses associated with control of viremia. Science 1997; 278(5342):1447-50.

224. Rossi M, Young JW. Human dendritic cells: potent antigen-presenting cells at the crossroads of innate and adaptive immunity. J Immunol 2005;175(3):1373-81.

225. Rouard H, Marquet J, Leon A, Maison P, Haioun C, Copie-Bergman C, et al. IL-12 secreting dendritic cells are required for optimum activation of human secondary lymphoid tissue T cells. J Immunother (1997) 2002; 25(4):324-33.

226. Rowell JF, Ruff AL, Guarnieri FG, Staveley-O'Carroll K, Lin X, Tang J, et al. Lysosome-associated membrane protein-1-mediated targeting of the HIV-1-1 envelope protein to an endosomal/lysosomal compartment enhances its presentation to MHC class II-restricted T cells. J Immunol 1995;155(4):1818-28.

227. Rowland-Jones S, Tan R, McMichael A. Role of cellular immunity in protection against HIV-1 infection. Adv Immunol 1997; 65:277-346.

228. Ruff AL, Guarnieri FG, Staveley-O'Carroll K, Siliciano RF, August JT. The enhanced immune response to the HIV-1 gp160/LAMP chimeric gene product targeted to the lysosome membrane protein trafficking pathway. J Biol Chem 1997; 272(13):8671-8.

229. Saeboe-Larssen S, Fossberg E, Gaudernack G. mRNA-based electrotransfection of human dendritic cells and induction of cytotoxic T lymphocyte responses against the telomerase catalytic subunit (hTERT). Journal of Immunological Methods 2002;259(1-2):191-203.

230. Sailaja G, Skountzou I, Quan FS, Compans RW, Kang SM. Human immunodeficiency virus-like particles activate multiple types of immune cells. Virology 2007; 362(2):331-41.

231. Sallusto F, Lanzavecchia A. Mobilizing dendritic cells for tolerance, priming, and chronic inflammation. J Exp Med 1999; 189(4):611-4.

232. Sallusto F, Lanzavecchia A. Understanding dendritic cell and T-lymphocyte traffic through the analysis of chemokine receptor expression. Immunol Rev 2000; 177:134-40.

233. Santra S, Schmitz JE, Kuroda MJ, Lifton MA, Nickerson CE, Lord CI, et al. Recombinant canarypox vaccine-elicited CTL specific for dominant and subdominant simian immunodeficiency virus epitopes in rhesus monkeys. J Immunol 2002; 168(4):1847-53.

234. Schaft N, Dorrie J, Thumann P, Beck VE, Muller I, Schultz ES, et al. Generation of an optimized polyvalent monocyte-derived dendritic cell vaccine by transfecting defined RNAs after rather than before maturation. J Immunol 2005; 174(5):3087-97.

235. Schmitz JE, Kuroda MJ, Santra S, Sasseville VG, Simon MA, Lifton MA, et al. Control of viremia in simian immunodeficiency virus infection by CD8+ lymphocytes. Science 1999;283(5403):857-60.

236. Schoenberger SP, Toes RE, van der Voort EI, Offringa R, Melief CJ. T-cell help for cytotoxic T lymphocytes is mediated by CD40-CD40L interactions. Nature 1998; 393(6684):480-3.

237. Schuler G, Schuler-Thurner B, Steinman RM. The use of dendritic cells in cancer immunotherapy. Curr Opin Immunol 2003; 15(2):138-47.

238. Schultz AM, Bradac JA. The HIV-1 vaccine pipeline, from preclinical to phase III. AIDS 2001; 15 Suppl 5:S147-58.

239. Serghides L, Bukczynski J, Wen T, Wang C, Routy JP, Boulassel MR, et al. Evaluation of OX40 ligand as a costimulator of human antiviral memory CD8 T cell responses: comparison with B7.1 and 4-1BBL. J Immunol 2005;175(10):6368-77.

240. Shedlock DJ, Shen H. Requirement for CD4 T cell help in generating functional CD8 T cell memory. Science 2003; 300(5617):337-9.

241. SHIV-1er JW, Emini EA. Recent advances in the development of HIV-1-1 vaccines using replication-incompetent adenovirus vectors. Annu Rev Med 2004; 55:355-72.

242. Simon F, Mauclere P, Roques P, Loussert-Ajaka I, Muller-Trutwin MC, Saragosti S, et al. Identification of a new human immunodeficiency virus type 1 distinct from group M and group O. Nat Med 1998;4(9):1032-7.

243. So T, Lee SW, Croft M. Tumor necrosis factor/tumor necrosis factor receptor family members that positively regulate immunity. Int J Hematol 2006; 83(1):1-11.

244. Spearman P. Current progress in the development of HIV-1 vaccines. Curr Pharm Des 2006; 12(9):1147-67.

245. Spiegel HM, Ogg GS, DeFalcon E, Sheehy ME, Monard S, Haslett PA, et al. Human immunodeficiency virus type 1- and cytomegalovirus-specific cytotoxic T lymphocytes can persist at high frequency for prolonged periods in the absence of circulating peripheral CD4(+) T cells. J Virol 2000; 74(2):1018-22.

246. Steinman RM, Pope M. Exploiting dendritic cells to improve vaccine efficacy. J Clin Invest 2002; 109(12):1519-26.

247. Steitz J, Bruck J, Knop J, Tuting T. Adenovirus-transduced dendritic cells stimulate cellular immunity to melanoma via a CD4(+) T cell-dependent mechanism. Gene Ther 2001; 8(16):1255-63.

248. Strobel I, Berchtold S, Gotze A, Schulze U, Schuler G, Steinkasserer A. Human dendritic cells transfected with either RNA or DNA encoding influenza matrix protein M1 differ in their ability to stimulate cytotoxic T lymphocytes. Gene Ther 2000; 7(23):2028-35.

249. Su Z, Dannull J, Heiser A, Yancey D, Pruitt S, Madden J, et al. Immunological and clinical responses in metastatic renal cancer patients vaccinated with tumor RNA-transfected dendritic cells. Cancer Res 2003; 63(9):2127-33.

250. Su Z, Dannull J, Yang BK, Dahm P, Coleman D, Yancey D, et al. Telomerase mRNA-transfected dendritic cells stimulate antigen-specific CD8+ and CD4+ T cell responses in patients with metastatic prostate cancer. Journal of Immunology 2005; 174(6):3798-807.

251. Su Z, Vieweg J, Weizer AZ, Dahm P, Yancey D, Turaga V, et al. Enhanced induction of telomerase-specific CD4(+) T cells using dendritic cells transfected with RNA encoding a chimeric gene product. Cancer Research 2002; 62(17):5041-8.

252. Sun JC, Bevan MJ. Defective CD8 T cell memory following acute infection without CD4 T cell help. Science 2003; 300(5617):339-42.

253. Taraban VY, Rowley TF, O'Brien L, Chan HT, Haswell LE, Green MH, et al. Expression and costimulatory effects of the TNF receptor superfamily members CD134 (OX40) and CD137 (4-1BB), and their role in the generation of anti-tumor immune responses. Eur J Immunol 2002;32(12):3617-27.

254. Tesselaar, K., R. Arens, G.M. van Schijndel, P.A. Baars, M.A. van der Valk, J. Borst, M.H. van Oers, and R.A. van Lier. 2003. Lethal T cell immunodeficiency induced by chronic costimulation via CD27-CD70 interactions. *Nat. Immunol.* 4:49–54.

255. Thery C, Amigorena S. The cell biology of antigen presentation in dendritic cells. Curr Opin Immunol 2001; 13(1):45-51.

256. Thomas R, Chambers M, Boytar R, Barker K, Cavanagh LL, MacFadyen S, et al. Immature human monocyte-derived dendritic cells migrate rapidly to draining lymph nodes after intradermal injection for melanoma immunotherapy. Melanoma Res 1999 ;9(5):474-81.

257. Thornburg C, Boczkowski D, Gilboa E, Nair SK. Induction of cytotoxic T lymphocytes with dendritic cells transfected with human papillomavirus E6 and E7 RNA: implications for cervical cancer immunotherapy. J Immunother (1997) 2000; 23(4):412-8.

258. Tseng WC, Haselton FR, Giorgio TD. Transfection by cationic liposomes using simultaneous single cell measurements of plasmid delivery and transgene expression. J Biol Chem 1997; 272(41):25641-7.

259. Turville SG, Cameron PU, Handley A, Lin G, Pohlmann S, Doms RW, et al. Diversity of receptors binding HIV-1 on dendritic cell subsets. Nat Immunol 2002; 3(10):975-83.

260. Turville SG, Santos JJ, Frank I, Cameron PU, Wilkinson J, Miranda-Saksena M, et al. Immunodeficiency virus uptake, turnover, and 2-phase transfer in human dendritic cells. Blood 2004; 103(6):2170-9.

261. Tuting T, Wilson CC, Martin DM, Baar J, DeLeo A, Lotze MT, et al. DNA vaccines targeting dendritic cells for the immunotherapy of cancer. Adv Exp Med Biol 1998; 451:295-304.

262. Tuting T, Wilson CC, Martin DM, Kasamon YL, Rowles J, Ma DI, et al. Autologous human monocyte-derived dendritic cells genetically modified to express melanoma antigens elicit primary cytotoxic T cell responses in vitro: enhancement by cotransfection of genes encoding the Th1-biasing cytokines IL-12 and IFN-alpha. J Immunol 1998; 160(3):1139-47.

263. Tuyaerts S, Michiels A, Corthals J, Bonehill A, Heirman C, de Greef C, et al. Induction of Influenza Matrix Protein 1 and MelanA-specific T lymphocytes in vitro using mRNA-electroporated dendritic cells. Cancer Gene Ther 2003; 10(9):696-706.

264. Vajdy M, Gardner J, Neidleman J, Cuadra L, Greer C, Perri S, et al. Human immunodeficiency virus type 1 Gag-specific vaginal immunity and protection after local immunizations with sindbis virus-based replicon particles. J Infect Dis 2001; 184(12):1613-6.

265. Van De Parre TJ, Martinet W, Schrijvers DM, Herman AG, De Meyer GR. mRNA but not plasmid DNA is efficiently transfected in murine J774A.1 macrophages. Biochem Biophys Res Commun 2005; 327(1):356-60.

266. Van Gulck ER, Ponsaerts P, Heyndrickx L, Vereecken K, Moerman F, De Roo A, et al. Efficient stimulation of HIV-1-1-specific T cells using dendritic cells electroporated with mRNA encoding autologous HIV-1-1 Gag and Env proteins. Blood 2006; 107(5):1818-27.

267. van Kooyk Y, Geijtenbeek TB. DC-SIGN: escape mechanism for pathogens. Nat Rev Immunol 2003; 3(9):697-709.

268. van Leeuwen EB, Cloosen S, Senden-Gijsbers BL, Germeraad WT, Bos GM. Transduction with a fiber-modified adenoviral vector is superior to non-viral nucleofection for expressing tumor-associated Ag mucin-1 in human DC. Cytotherapy 2006; 8(1):36-46.

269. Van Tendeloo VF, Ponsaerts P, Lardon F, Nijs G, Lenjou M, Van Broeckhoven C, et al. Highly efficient gene delivery by mRNA electroporation in human hematopoietic cells: superiority to lipofection and passive pulsing of mRNA and to electroporation of plasmid cDNA for tumor antigen loading of dendritic cells. Blood 2001; 98(1):49-56.

270. Van Tendeloo VF, Snoeck HW, Lardon F, Vanham GL, Nijs G, Lenjou M, et al. Nonviral transfection of distinct types of human dendritic cells: high-efficiency gene transfer by electroporation into hematopoietic progenitor- but not monocyte-derived dendritic cells. Gene Ther 1998; 5(5):700-7.

271. Veazey RS, Tham IC, Mansfield KG, DeMaria M, Forand AE, Shvetz DE, et al. Identifying the target cell in primary simian immunodeficiency virus (SIV) infection: highly activated memory CD4(+) T cells are rapidly eliminated in early SIV infection in vivo. J Virol 2000; 74(1):57-64.

272. Vidalin O, Tanaka E, Spengler U, Trepo C, Inchauspe G. Targeting of hepatitis C virus core protein for MHC I or MHC II presentation does not enhance induction of immune responses to DNA vaccination. DNA Cell Biol 1999; 18(8):611-21.

273. Villamide-Herrera L, Ignatius R, Eller MA, Wilkinson K, Griffin C, Mehlhop E, et al. Macaque dendritic cells infected with SIV-recombinant canarypox ex vivo induce SIV-specific immune responses in vivo. AIDS Res Hum Retroviruses 2004; 20(8):871-84.

274. Walker BD, Korber BT. Immune control of HIV-1: the obstacles of HLA and viral diversity. Nat Immunol 2001; 2(6):473-5.

275. Walsh SR, Bhardwaj N, Gandhil RT. Dendritic cells and the promise of therapeutic vaccines for human immunodeficiency virus (HIV-1)-1. Curr HIV-1 Res 2003; 1(2):205-16.

276. Watts TH. TNF/TNFR family members in costimulation of T cell responses. Annu Rev Immunol 2005; 23:23-68.

277. Wei X, Decker JM, Wang S, Hui H, Kappes JC, Wu X, et al. Antibody neutralization and escape by HIV-1-1. Nature 2003; 422(6929):307-12.

278. Weissman D, Fauci AS. Role of dendritic cells in immunopathogenesis of human immunodeficiency virus infection. Clin Microbiol Rev 1997; 10(2):358-67.

279 Weissman D, Ni H, Scales D, Dude A, Capodici J, McGibney K, et al. HIV-1 gag mRNA transfection of dendritic cells (DC) delivers encoded antigen to MHC class I and II molecules, causes DC maturation, and induces a potent human in vitro primary immune response. Journal of Immunology 2000; 165(8):4710-7.

280. Wolthers KC, Otto SA, Lens SM, Kolbach DN, van Lier RA, Miedema F, et al. Increased expression of CD80, CD86 and CD70 on T cells from HIV-1-infected individuals upon activation in vitro: regulation by CD4+ T cells. Eur J Immunol 1996; 26(8):1700-6.

281. Wu L, KewalRamani VN. Dendritic cell interactions with HIV-1: infection and viral dissemination. Nat Rev Immunol 2006; 6(11): 859-68.

282. Wu TC, Guarnieri FG, Staveley-O'Carroll KF, Viscidi RP, Levitsky HI, Hedrick L, et al. Engineering an intracellular pathway for major histocompatibility complex class II presentation of antigens. Proc Natl Acad Sci U S A 1995; 92(25):11671-5.

283. Yewdell JW, Del Val M. Immunodominance in TCD8+ responses to viruses: cell biology, cellular immunology, and mathematical models. Immunity 2004; 21(2):149-53.

284. Younes SA, Yassine-Diab B, Dumont AR, Boulassel MR, Grossman Z, Routy JP, et al. HIV-1-1 viremia prevents the establishment of interleukin 2-producing HIV-1-specific memory CD4+ T cells endowed with proliferative capacity. J Exp Med 2003; 198(12):1909-22.

285. Yu Q, Yue FY, Gu XX, Schwartz H, Kovacs CM, Ostrowski MA. OX40 ligation of CD4+ T cells enhances virus-specific CD8+ T cell memory responses independently of IL-2 and CD4+ T regulatory cell inhibition. J Immunol 2006; 176(4):2486-95.

286. Yu XG, Addo MM, Rosenberg ES, Rodriguez WR, Lee PK, Fitzpatrick CA, et al. Consistent patterns in the development and immunodominance of human immunodeficiency virus type 1 (HIV-1-1)-specific CD8+ T-cell responses following acute HIV-1-1 infection. J Virol 2002; 76(17):8690-701.

287. Zajac AJ, Blattman JN, Murali-Krishna K, Sourdive DJ, Suresh M, Altman JD, et al. Viral immune evasion due to persistence of activated T cells without effector function. J Exp Med 1998;188(12):2205-13.

288. Zhang L, He T, Huang Y, Chen Z, Guo Y, Wu S, et al. Chemokine coreceptor usage by diverse primary isolates of human immunodeficiency virus type 1. J Virol 1998; 72(11):9307-12.

289. Zhang M, Gaschen B, Blay W, Foley B, Haigwood N, Kuiken C, et al. Tracking global patterns of N-linked glycosylation site variation in highly variable viral glycoproteins: HIV-1, SIV, and HCV envelopes and influenza hemagglutinin. Glycobiology 2004; 14(12):1229-46.

290. Zhang Y, Lou B, Lal RB, Gettie A, Marx PA, Moore JP. Use of inhibitors to evaluate coreceptor usage by simian and simian/human immunodeficiency viruses and human immunodeficiency virus type 2 in primary cells. J Virol 2000; 74(15):6893-910.

291. Zhang Z, Schuler T, Zupancic M, Wietgrefe S, Staskus KA, Reimann KA, et al. Sexual transmission and propagation of SIV and HIV-1 in resting and activated CD4+ T cells. Science 1999; 286(5443):1353-7.

292. Zhong L, Granelli-Piperno A, Choi Y, Steinman RM. Recombinant adenovirus is an efficient and non-perturbing genetic vector for human dendritic cells. Eur J Immunol 1999;29(3): 964-72.