ABSTRACT

Development of Dendritic Cell-based Vaccines for the Treatment of HIV-Infected Patients

Amanda J. Cobb, Ph.D.

Mentor: Karolina Palucka, M.D. Ph.D.

Dendritic cells (DCs) are professional antigen-presenting cells of the immune system with the ability to induce and control T and B cells responses. Monocytes have been used as precursors to generate DC vaccines ex vivo. Monocyte-derived DCs have been used as vaccines in clinical trials to treat cancer and infectious disease by eliciting potent T cell responses. Infection with Human Immunodeficiency Virus (HIV) almost invariably leads to a chronic disease. Eventually the destruction of the immune system culminates into Acquired Immune Deficiency Syndrome (AIDS). Since the advent of Highly Active Antiretroviral Therapy (HAART), HIV patients have been able to control viral levels. However, with prolonged use of treatment, these patients experience serious adverse effects such as liver and mitochondrial toxicity that can potentially become fatal.

Long-Term Non-Progressors (LTNP) are a unique subpopulation of HIV patients that are able to control their viral load without HAART. Studies revealed HIV-specific polyfunctional $CD8^+$ T cells to be vital for viral control. Thus, we have focused our efforts on devising therapeutic HIV DC vaccines to increase the quality of $CD8^+$ T cell responses in chronic HIV infected patients. First, we conducted a series of preclinical

development assays of GM-CSF/IFN-α and GM-CSF/IL-15 HIV DC vaccines loaded with a mixture of HIV-1-antigen lipopeptides (ANRS HIV-LIPO-5 vaccine), which is comprised of five HIV-1-antigen peptides (Gag_{17-35}, $Gag_{253-284}$, Nef_{66-97}, $Nef_{116-145}$, and $Pol_{325-355}$) each covalently linked to a palmitoyl-lysylamide moiety, and activated with LPS. Functional assays demonstrated that the DC vaccines expressed DC differentiation markers and displayed typical morphology which included the presence of dendrites important for cellular interaction. Potency assays proved the DC vaccines were capable of eliciting HIV-1-antigen-specific $CD4^+$ and $CD8^+$ T cell responses.

Our studies have demonstrated the use of DC vaccines in eliciting HIV-specific $CD8^+$ T cells responses in vitro. Furthermore, the pre-clinical development of the GM-CSF/IFN-α DC vaccine activated with LPS led to the evaluation of safety and immunogenicity in a Phase I/II clinical trial in chronically HIV-1-infected patients on HAART (clinicaltrials.gov identifier: NCT00796770).

Development of Dendritic Cell-based Vaccines for the Treatment of HIV-Infected Patients

by

Amanda J. Cobb, B.S.

A Dissertation

Approved by the Institute of Biomedical Studies

_____
Robert R. Kane, Ph.D., Chairperson

Submitted to the Graduate Faculty of
Baylor University in Partial Fulfillment of the
Requirements for the Degree
of
Doctor of Philosophy

Approved by the Dissertation Committee

_____
A. Karolina Palucka, M.D., Ph.D., Chairperson

_____
Jacques F. Banchereau, Ph.D.

_____
Gerard Zurawski, Ph.D.

_____
Christopher Kearney, Ph.D.

Accepted by the Graduate School
August 2011

_____
J. Larry Lyon, Ph.D., Dean

Page bearing signatures is kept on file in the Graduate School.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF SCHEMES

LIST OF TABLES

LIST OF ABBREVIATIONS

AIDS – Acquired immunodeficiency syndrome

AA – Amino acids

ANRS – Agence Nationale de Recherché sur le SIDA (French National Agency for AIDS Research)

APC – Antigen presenting cells

CFSE – Carboxyfluorescein succinimidyl ester

cRPMI – Complete RPMI 1640 with 2.5% hepes, 1% penicillin/streptomycin, 1% non-essential amino acids, 1% L-glutamine, and 0.1% β-mercaptoethanol, supplemented with 10% human serum type AB

C- – Control negative

C+ – Control positive

CTL – Cytotoxic $CD8^+$ T cells

DC – Dendritic cells

DMSO – Dimethyl sulfoxide

FACS – Flow cytometry

GM-CSF – Granulocyte-macrophage colony-stimulatory factor

HAART – Highly active antiretroviral therapy

HC – HIV-1 controllers

HIV-1 – Human immunodeficiency virus type-1

IFN – Interferon

IL – Interleukin

IONO – Ionomycin

LIPO-5 – ANRS HIV-1-antigen lipopeptides: Gag_{17-35}, $Gag_{253-284}$, Nef_{66-97}, $Nef_{116-145}$, and $Pol_{325-355}$ peptides that are covalently linked at their C-terminal end to a palmitoyl-lysylamide moiety

LPS – Lipopolysaccharide

LTNP – Long-term non-progressors

MLR – Mixed lymphocyte reaction

PBMC – Peripheral blood mononuclear cells

PMA – Phorbol 12-myristate 13-acetate

rhCD40L – Recombinant-human CD40 ligand

ACKNOWLEDGMENTS

First, I would like to thank my former mentor, Jacques Banchereau for his guidance and encouragement to pursue a further understanding of science. He has been an integral part of my development in the last 5 years and has taught me how to form the essential questions to answer in science. He also provided me the opportunity to train with world-renowned scientists. I kindly thank the guidance and dedication of Karolina Palucka, my mentor, to my growth as a scientist over the last 6.5 years. I would like to thank my committee members, Dr. Gerard Zurawski, Dr. Christopher Kearney, Dr. Robert Kane, and Dr. Cheolho Sim for their commitment to my education. With much appreciation and thanks, Lee Roberts has been an integral part of my development into a scientist and writer. He has guided me throughout these last 6.5 years in writing technical reports and discussions. I would like to thank our collaborators from the French National Agency for AIDS Research (ANRS) Paris, France, in particular, Dr. Yves Levy for his continued support in my education and in HIV vaccine research. I would very much like to thank Susan Burkeholder for her mentoring of the art of dendritic cell biology, patience, and encouragement. I would like to thank Dr. Monica Montes, Rajaram Ranganathan, Anne-Laure Flamar, Dr. Jason Skinner, Nicolas Loof and Kay Kayembe for the formation of an incredibly passionate group dedicated to the quest of developing a therapeutic HIV vaccine. I most definitely appreciate and thank Dr. Derek Blankenship for his help with statistical analysis of my data. I would like to thank Dr. Eynav Klechevsky for her help in understanding how to assess my DC vaccines in vitro with T cells. I would like to thank Dr. Luann Snipes for her discussions and guidance.

would also like to thank the core facilities for their expertise including, Lynnette Walters and Michelle Hughes in the Sample Core; Elizabeth Trahan and Sebastian Coquery in the Flow Core; Sandra Clayton in the Microscope Core; Charles McWilliams, Jennifer Finholt, and Jill Plants in the GMP Core; and Sandy Zurawski, Jill Plants, and Olivier Agouna-Deciat in the Luminex Core. I thank Bryan King, Dr. Louis Sloan and the medical staff from North Texas Infectious Disease Consultants for their devotion to HIV vaccine clinical studies. I would also like to thank the volunteers for participation in this study. I would like to thank the financial support of BHCS Foundation which made this research possible. I would like to thank Dr. Michael Ramsay and the administrative staff from both Baylor University at Waco and Baylor Institute for Immunology Research including Rhonda Bellert, Cindy Samuelsen, Dr. Carson Harrod, David Schackmann, Angela Plata, and Dorcus Simmons for their assistance in helping to coordinate coursework, presentations, grants and publications.

DEDICATION

To Him first who saved, provided and guided my way; my beloved husband for his love, patience and encouragement; my daughter for motivation and inspiration; my father and mother for my love of science; my mother- and father-in-law for their support; my brother, sister and sister-in-law; and finally my mentors for their teaching, guiding, and correction along the way

CHAPTER ONE

Introduction

Human Immunodeficiency Virus (HIV)

History and Background

The early 1980's proved to be a time of discovery for retrovirologists. After a number of recreational intravenous drug users and homosexual men presented with symptoms of *Pneumocystic carinii* pneumonia, the Centers for Disease Control (CDC) coined the term Acquired Immuno-Deficiency Syndrome (AIDS) in a newsletter released June 5, 1981 (Gottlieb 2006; Gottlieb and others 1981). This was a rare infection only seen in patients with severely compromised immune systems. Additionally, cases of Kaposi's sarcoma were becoming increasingly common in the Los Angeles and Orange County regions of California. These events led to a task force headed by the CDC to investigate and monitor the disease outbreaks (1982).

The race was on for three research laboratories to identify the causative agent for AIDS. The labs were headed up by Luc Montagnier, Robert Gallo, and Jay Levy. In 1983, French virologist, Françoise Barré-Sinoussi along with Luc Montagnier isolated a lymph node from a patient with AIDS. They observed that the virus within the lymph node contained reverse transcriptase, a typical enzyme of retroviruses, and deemed that this was the cause of AIDS (Barre-Sinoussi and others 1983). They named the newly discovered pathogen based on the site of discovery, lymphadenopathy-associated virus (LAV) (Montagnier and others 1984). In early 1984, Robert Gallo and Jay Levy's

laboratories also identified similar viruses they claimed were the cause of AIDS. Gallo termed their virus human T cell leukemia virus (HTLV)-III (Gallo and others 1984; Popovic and others 1984) and Levy termed their virus AIDS-associated retrovirus (ARV) (Levy and others 1984). The viruses from these three laboratories, LAV, HTLV-III and ARV, were found to be similar and were indeed the cause of AIDS (Ratner and others 1985). The National Institutes of Health (NIH) later found these viruses to be from the same retroviral family as well as part of a smaller subset group called lentivirus (Chermann and others 1991; Guo and others 1991). However, after consultation with the International Committee of Taxonomy of Viruses it was suggested the virus should have a separate name. HIV was then devised (Coffin and others 1986).

Over the course of time, HIV infections have reached pandemic levels as 33.3 million people were reported to be living with HIV in the year of 2009 according to the World Health Organization (WHO) (Zarocostas 2010). Of these 33.3 million people, the largest population of 68% was living in Sub-Saharan Africa, the second largest population of 12% living in South and South-east Asia and the third largest population of 5% living in North America. In that same year, 1.8 million HIV-related deaths were reported. Of the 1.8 million deaths, Sub-Saharan Africa claimed 72% which was the largest amount of deaths and North America claimed 14% of deaths which amounted to be the sixth largest population (Zarocostas 2010).

HIV Physical Structure

The structure of HIV consists of 72 knobs residing on the external surface known as the envelope glycoproteins, gp120 and gp41, of the virion (Earl and others 1990; Ozel and others 1988). The virion is enveloped with a lipid bilayer supporting the knobs.

Inside the lipid bilayer there is a capsid protein protecting the core called myristoylated p17 core protein (MA) (Gelderblom and others 1989). MA protects the integrity of the virion components in the core which consist of Vpr and p24 (Lu and others 1993; Yu and others 1993). However, p24 in itself contains the information needed for the virion to integrate into the host genome after gaining entry into host cell. p24 houses the two full-length viral messenger RNA strands, that codes for Gag and Pol proteins, as well as reverse transcriptase and nucleocapsid proteins (Levy 1994).

The HIV envelop, Env gp160, has been studied extensively due to the infective interaction with $CD4^+$ T cells. It is broken up into gp120 and gp41. Gp120 is an external surface envelope protein while gp41 is a transmembrane protein. Both proteins are responsible for virion: cell fusion/infection. Observed by electron microscopy, the ectodomain of gp41 is observed to be organized as a trimer lending to the ability of the cellular fusion (Earl and others 1990; Weiss and others 1990).

Analysis of an African serum sample from 1959 marks the first evidence of HIV infection in humans (Nahmias and others 1986). Since then viral isolates have been collected from patients and extensively studied. Among the assays included sequencing the viral genome. Comparing sequences to patients in different areas of the world, scientists found the virus to be quite heterogeneous. It has evolved overtime creating several different strains and within those strains several different subtypes, also known as clades (Pantaleo and Walker 2001). There are two types of HIV; HIV-1 and HIV-2. HIV-1 is classified into 4 strains, M or “major”, O or “outlier”, N and P. Within HIV-1 strain M, 9 genetically different clades are seen known as A, B, C, D, F, G, H, J and K.

HIV-2 is isolated to West Africa and has a lower infectivity rate (Levy 1994). The HIV-1 strain M clade B is the main focus of our study and will be referred to as HIV.

Clinical Manifestations

Infection with HIV is transmitted through contaminated needle-sharing, occupational accidental contaminated needle-sticks, sexual intercourse, or from HIV infected mother to child. During the first weeks of infection, also known as the acute phase, patients develop many flu-like symptoms such as fever, headache, pneumonitis, diarrhea, and enlarged lymph nodes (Cooper and others 1985; Tindall and others 1988). Laboratory tests performed in the acute phase reveal a sharp spike in $CD8^+$ T cells, a decrease of $CD4^+$ T cells thus causing an inverted $CD4^+$:$CD8^+$ T cell ratio, anti-HIV antibodies, and a detectable viral load (Cooper and others 1988). However, after 1-3 weeks, the flu-like symptoms dissipate as the patient enters the asymptomatic phase. This phase can last for months and even years accompanied by a steady decline of $CD4^+$ T cells ((Lang and others 1989)).

On the cellular level, infection with HIV causes many dysfunctions within the immune system (Catalfamo and others 2008). The virus utilizes the host's surface protein CD4 as the main receptor (Dalgleish and others 1984; Klatzmann and others 1984a; Klatzmann and others 1984b) and requires a co-receptor, chemokine receptor type-4, CXCR-4 (Berson and others 1996; Doranz and others 1996) or CCR-5 (Rucker and others 1996; Trkola and others 1996) for cellular fusion and entry. $CD4^+$ T cells bear CD4 and CXCR-4 as well as CCR-5 which is the favored cellular target of HIV while macrophages bear CD4 and CCR-5 and represent another cellular target of the virus. Due to the infection of $CD4^+$ T cells during the acute phase of infection, research has

shown these dysfunctional cells lead to impairment in the recruitment of cytotoxic T lymphocytes (CTL) against virus-infected cells (Margolick and others 1995).

Additional immune cells help to facilitate the transfer of HIV by their expression of Fc receptors (Homsy and others 1989; Laurence and others 1990; Takeda and others 1988). In an attempt by the immune system to rid the body of the virus, immune complement coats the virion. The complement-coated virions then 'hitch a ride' via cells expressing Fc receptors to the lymph node. Once in the lymph node, the virion then uses the same receptor/co-receptor to gain entry to cells that contain the necessary machinery to replicate. Viral reservoirs are established by the infection and integration of HIV into $CD4^+$ T cells that reside in the lymph tissue, including the gut-associated lymphoid tissue (Chun and Fauci 1999).

Not all individuals infected with HIV present with the same symptoms. Thus, patients are categorized depending on anti-HIV antibodies, viral titre, and $CD4^+$ T cell counts (1990). The majority of the HIV infected population in North America and Europe fall into the group of chronically infected HIV individuals. In this group, anti-HIV antibodies are present but due to the high rate of mutation of the virus, they are not protective, viral titre is high, and there is continued death of the $CD4^+$ T cells causing their peripheral blood count to fall below 200 cells/µL vs. ~1,300±450 cells/µL in normal individuals. This extremely low $CD4^+$ T cells count is the benchmark which defines AIDS (Castro and others 1992). AIDS is clinically characterized by a significant decrease in $CD4^+$ T cells and the development of opportunistic infections and cancers due to uncontrolled viral replication of HIV (Pantaleo and Walker 2001). There is, however, a unique sub-group within the HIV infected patient population (called long-term non-

progressors (LTNP), or elite controllers) that have the ability to control their viral load (reviewed in (Deeks and Walker 2007)). They have a small subset of antigen-specific polyfunctional CTL that are able to kill infected cells. This distinct population will be expounded upon later in this text.

Conventional Therapy

HIV is currently incurable but it is treatable with highly active anti-retroviral therapy drugs, which are able to keep the viral load undetectable. In the last decade, the advent of highly active antiretroviral therapies (HAART) that contain HIV replication has changed the course of HIV infection by reducing the AIDS-related morbidity and mortality of patients (Virgin and Walker 2010). This clinical benefit is clearly related to limiting the immunological damage that is caused by HIV replication as well as to the restoration of $CD4^+$ T lymphocyte counts and specific responses against pathogens (Kaufmann and others 2000; McMichael and others 2010).

HAART consists of a combination of antiretroviral drugs composed of nucleoside/nucleotide analogue reverse transcriptase inhibitors and either a non-nucleoside reverse transcriptase inhibitor or a protease inhibitor. HAART is efficient at suppressing the replication of HIV to undetectable levels, which corresponds to an increase of $CD4^+$ T cell counts in treated patients (Shen and Siliciano 2008).

However, despite the therapeutic effect of HAART, there are several concerns that are now arising. The goal of HAART initially was to irradicate the virus totally from the host. Studies conducted by Shen & Siliciano reveal the virus is still replicating in reservoirs at an undetectable level. They conclude the limits of HAART therapy might fall short of irradication due to the HIV reservoirs (Shen and Siliciano 2008).

Furthermore, patients medicated with HAART for a prolonged period of time, eventually start to show signs of substantial toxic side effects (Buchacz and others 2008; Groopman 1990). These side effects encompass liver and neurological toxicities (Carter 2003; Millogo and others 2008; Soriano and others 2008). The toxicities of HAART also accompany a financial burden. The cost to stay on HAART ranges from $10,000-$20,000 per year (Hill and Smith 2007). Once the treatment has begun, interruption in therapy has been thought to cause resistant (Dybul and others 2002). With all the above mentioned concerns, restoration of the adaptive immune response against HIV is at the heart of immunotherapy research in hope of controlling viral replication and limiting drug exposure.

Overview of the Immune System

Immunology is the study of all aspects of a host's defense against infection and of adverse consequences of immune responses. Without a functional immune system, one could not survive an infection. The immune system provides several layers of protection. First, it can recognize damaged or aged cells and silently eliminate them. Secondly, it can launch defenses against bacterial, parasitic, fungal, and viral invaders. Thirdly, it kills tumor cells. An overly active immune system can result in allergies or autoimmune disease. A hypoactive immune system can result in chronic infection or cancer.

The immune system is composed of different types of cells such as T cells, B cells, natural killer (NK) cells, monocytes, dendritic cells (DC), mast cells, eosinophils, basophils, and macrophages. It is divided into two different branches of response. The first branch is the innate immune response. This response is not specific and happens soon after e.g., microbial invasion. It consists of the epithelial barrier, cells that combat

invading pathogens (i.e., macrophages and natural killer cells), cellular receptors that recognize pathogens such as pathogen associated molecular patterns and toll like receptors (TLR), complement pathways which mediate humoral innate immunity, and antimicrobial proteins released from cells to aggravate a microbe's capacity to establish an infection. The second division is the adaptive immune response. This response happens when the microbe breeches the innate immune response. It is a specific response and takes time to establish. In an adaptive immune response there are two distinct arms. One arm is the cellular immunity which consists of cells attacking the microbe and the other is humoral immunity in which antibodies produced from B cells attach themselves to the microbe tagging the microbe for destruction, or preventing it's transfer to new host cells. Depending on what type of microbe is present, one or both arms of the adaptive immune system could be called into action.

T Cells

T lymphocytes are distinctively divided into $CD8^+$ and $CD4^+$ T cells. $CD8^+$ T cells, also known as CTLs, have the ability to kill cells that are infected or dysfunctional. $CD4^+$ T cells provide the help to sustain and regulate the cytotoxic effects of the CTLs. $CD4^+$ T cells are divided further by their specific characteristics exemplified by particilar effector functions. Thus, $CD4^+$ Th1 T cells secrete interleukin (IL)-2, interferon (IFN)-γ, and tumor necrosis factor (TNF)-α that helps at eliciting strong cellular immune responses. However, $CD4^+$ Th2 T cells secrete IL-4, IL-5, IL-6, IL-10, and IL-13 which helps in the development of the humoral immune responses (Mosmann and Sad 1996).

The programming of the T cells to engage in such actions depends on the information given by the DCs. Using peptide binding grooves established by major

histocompatibility complex (MHC)-I or II, $CD8^+$ T cells or $CD4^+$ T cells are educated, respectively. Peptides that are presented in MHC I consist of 8-10 amino acids (AA). Peptides that are presented in the MHC II groove consist of 12-24 AA. Within the genes that encode MHC, there is a distinct set of genes that make up the human leukocyte antigen (HLA) composition of an individual. There are two alleles for each of the HLA molecules which are co-expressed. HLA molecules are divided into two groups. Class I is composed of HLA-A, -B, and -C molecules and recognized by $CD8^+$ T cells. Class II is composed of HLA-DR, -DP, and –DQ molecules and is recognized by $CD4^+$ T cells. The development of an antigen-specific T cell largely depends on the HLA molecules as well as environmental factors (Janeway 2005).

The MHC compartments are recognized by the T cells via T cell receptor (TCR). The TCR is a heterodimeric cell surface protein and is composed of α, β, γ, or δ chains with variable sequences in the regions that interact with the MHC peptide complex. The majority of the T cells in the periphery carry α and β chains. Following the interaction of the MHC peptide complex with a specific TCR, co-stimulation occurs via CD28 on T cells and B7 proteins on the DCs, such as CD80 and CD86 (Janeway 2005). During this cell-cell communication, the cytokines and chemokines released from the DCs can significantly influence the quality of the T cell responses. It has been reported that naïve $CD8^+$ T cells can be polarized to CTLs by the interaction with IL-12-secreting-DC (Banchereau and Palucka 2005).

In HIV, T cell populations are progressively devastated. $CD4^+$ T cells co-expressing CXCR-4 and CCR-5 are the prime target of HIV. During the first few weeks of infection, there is a dramatic drop in the $CD4^+$ T cell count as the HIV is in mass

production. The drop in $CD4^+$ T cells, in the majority of patients, negatively influences the quality of $CD8^+$ T cells to function as CTLs. Although $CD8^+$ T cells are seen in abundance in acute infection, this could be the result of the "cytokine storm" and overall chaos induced by the virus. After homeostasis is reached and patient enters the asymptomatic phase, $CD8^+$ T cells return to normal levels and $CD4^+$ T cells begin their decent as the virus continues to replicate and kill $CD4^+$ T cells.

Elite Controllers

A unique sub-group within the HIV infected patient population LTNP, or HC are able to control their viral load without the use of HAART therapy. LTNP make up 5-15% of HIV infected patients (Cao and others 1995) and HC make up less than 1% (reviewed in (Deeks and Walker 2007)). They have a small subset of HIV antigen-specific polyfunctional CTL expressing both effector cytokines (including IL-2) and the capability of degranulating to lyse HIV infected cells (Migueles and others 2008). Furthermore, studies in macaques have indeed shown that vaccines inducing $CD8^+$ T cells can result in a dramatic decrease of serum simian immunodeficiency virus levels (Bonaldo and others 2010; Liu and others 2009).

Borrowing from these observations, design of therapeutic vaccines for the treatment of HIV infected patients should be geared to increase the number and efficacy of these polyfunctional HIV-antigen-specific CTL, as well as improving $CD4^+$ T cell help, and NK lysing (McMichael and others 2010). The expansion of this polyfunctional CTL population would, thus, mimic the immunological status seen in LTNP. This approach could reduce patient-induced viral spreading and potentially eliminate the necessity of treating patients with HAART.

HIV Immunotherapy

The rationale for therapeutic immunization in HIV infection is based on several lines of evidence suggesting that the immune system contributes to the long-term control of HIV replication (Borrow and others 1994; Cao and others 1995; Deeks and Walker 2007; Koup and others 1994; Rosenberg and others 1997). Remarkably, a state of durable evolution of HIV infection without a significant decrease of $CD4^+$ T cell counts and/or detectable viral replication does occur in a LTNP or HC. These clinical observations provide clear evidence that durable containment of HIV replication and/or prevention of disease progression without antiretroviral therapy is possible. However, continued viral replication leading to progressive immune destruction is the rule in a majority of patients. In the long term, although HIV-specific $CD4^+$ and $CD8^+$ T cells may be detectable in patients at different time points of the disease, these cells are functionally impaired and fail to control viral replication after treatment discontinuation (Appay and others 2000; Carcelain and others 2001; Champagne and others 2001; Goepfert and others 2000). Therefore, the hope is that an immune and/or vaccine intervention might mobilize some of the mechanisms that mediate control of viral replication in these rare patients and, by doing so, control viral replication or lower the viral "set point" in patients who did not achieve this equilibrium on their own.

Current HIV immunotherapy protocols in patients receiving HAART utilize: i) cytokines to improve T cell function (Levy and others 2009; Pett 2009), ii) vaccines based on HIV lipopeptides (Durier and others 2006; Levy and others 2005), iii) adenovirus type 5 (Ad5) vector-based vaccines encoding HIV genes (Buchbinder and others 2008; Catanzaro and others 2006; Priddy and others 2008), or iv) vaccines based

on ex-vivo generated dendritic cells (DCs) that are loaded with HIV antigens (from proteins Gag, Nef, Pol, and/or Env or whole inactivated virus (Carbonneil and others 2003; Lu and others 2004) which will be covered in a separate section.

Cytokine Therapy

In the majority of HIV infected patients, a gradual decline of both the memory and naïve $CD4^+$ T cells is seen. This decline becomes problematic when memory $CD4^+$ T cells for a particular microbe are depleted and the host is no longer protected. The lack of protection leads to opportunistic infections that thus plague the patients. This coupled with the dysfunction of immune system as a whole, eventually leads to death. Since 1983, restoring the levels of $CD4^+$ T cell counts is at the heart of cytokine therapy. Cytokines that have been the common focus of non-specific expansion of T cell pools include IL-2, IL-4, IL-7 and GM-CSF.

IL-2 is a 15 kDa protein and is produced by T cells. It was originally named T cell growth factor due to the proliferative effect it has on T lymphocytes. IL-2 is also a T cell proliferative factor and enhances cytolytic activity and induces antibody production as well as cytokine secretion. Because of these properties, IL-2 gained attention for the use in re-populating $CD4^+$ T cells and possibly enhancing the function of HIV CTLs and thus has been evaluated in clinical trials (Pett and others 2010). It was demonstrated in clinical trials that when IL-2 was administered for 5 days every 8 weeks, then $CD4^+$ T cell populations, both memory and naïve, were expanded (Abrams and others 2009). However, bursts in the viral load of HIV were also observed (Kovacs and others 2000). It was originally thought that these bursts were in response to the disruption of the latent viral reservoirs but research studies have yet to prove this theory.

IL-4 is a 17 kDa glycoprotein and is a Th2 type cytokine. It is produced by activated T cells and induces B cell growth as well as differentiation. IL-6 another cytokine produced by T cells and DCs induced Kaposi's sarcoma progression. IL-4 has been shown to have antagonistic effects on Kaposi's. Therefore, IL-4 became an interesting cytokine to study in HIV infected patients. Kaposi's sarcoma is commonly seen in late stages of infection when $CD4^+$ T cells counts are critically low. Thus, the majority of clinical trials were designed to target patients that have progressed to AIDS to study the potential therapeutic use of this cytokine. Studies have found that $CD4^+$ T cells are increased by this therapy, but only temporarily and plasma HIV viral loads are decreased (Pantaleo and Walker 2001).

IL-7 is hematopoietic growth factor cytokine. It is produced by a host of cells and influences T cell homeostasis (Fry and Mackall 2002a; 2002b). Studies have shown that IL-7 reactivates HIV replication in T cells infected with the provirus; thus possibly providing a mechanism for reducing the viral reservoir (Levy 2006; Levy and others 2009; Wang and others 2005). In mice, it has been demonstrated that the after vaccination with a recombinant lentivector/melan-A_{26-35} vaccine, the addition of IL-7 enhances the survival of antigen-specific effector $CD8^+$ T cells (Colombetti and others 2009). IL-7 is currently been evaluated in a Phase II clinical trial in conjunction with HAART.

GM-CSF is an 18 kDa glycoprotein. It is secreted by monocytes, fibroblasts, and endothelial cells. Regulating the production and function of neutrophils is the major function of this cytokine. In studies conducted in HIV patients with advanced disease, GM-CSF has been shown to indirectly increase the number of memory $CD4^+$ T cells.

This observation could be linked to what has been demonstrated in in vitro studies in which GM-CSF has been shown to induce cells to secrete IL-2 (Pantaleo and Walker 2001).

Although cytokine therapy can improve $CD4^+$ T cell populations and possesses the potential in reducing the risk of opportunistic infections, it has limitations. Cytokine therapy does not improve long term HIV memory responses. However, cytokine therapy could be useful in combinations with other immunotherapy methods to enhance immunity against HIV.

HIV LIPO-5 Peptides

Since 1994, the French National Agency for AIDS Research (ANRS) has studied conserved regions of HIV-1 for use as antigens in active immunotherapeutic vaccine clinical trials. Of these regions, five immunogenic peptides ranging from 19-32 amino acids in length were identified for inclusion in the ANRS HIV-LIPO-5 vaccine. The ANRS HIV-LIPO-5 vaccine is comprised of the $Gag_{17\text{-}35}$, $Gag_{253\text{-}284}$, $Nef_{66\text{-}97}$, $Nef_{116\text{-}145}$, and $Pol_{325\text{-}355}$ peptides that are covalently linked at their C-terminal ends to a palmitoyl-lysylamide moiety (Durier and others 2006; Levy and others 2005). Clinical trials in healthy volunteers and chronically HIV infected patients demonstrated immunogenicity of LIPO-5 (Goujard and others 2007; Launay and others 2007; Pialoux and others 2001; Pialoux and others 2008; Salmon-Ceron and others 2010). In a randomized trial conducted by the ANRS, 70 HIV infected adults were vaccinated with either excipient (controls) or four intramuscular injections of ALVAC-HIV and LIPO6T (lipopeptides with HIV peptide sequences) followed by three cycles of subcutaneous IL-2 while continuing HAART (Levy and others 2005). The breadth and the magnitude of HIV-

specific interferon-gamma-producing $CD8^+$ T cells were greater in the vaccinated group. After stopping HAART, 24% of the vaccinated group lowered their viral set point compared with 5% of controls.

This provided the proof-of-concept that vaccination prior to the removal of HAART may contribute to the lower set point of HIV replication (Levy and others 2005). As of 2006, the ANRS has used 4 different combinations of HIV lipopeptides for vaccinations in eleven clinical trials. Nine of these clinical trials were preventive (eight were conducted in France and one in the United States). Two clinical trials were therapeutic and conducted in France. Overall, 250 healthy uninfected or HIV infected adults were vaccinated in France and 125 healthy adults were vaccinated in the United States. Between 1 and 5 vaccinations were given either intramuscularly or intradermally, depending on the study. After an extensive meta-analysis of the trials to evaluate the safety of the lipopeptides, published results have reported that the lipopeptide vaccines demonstrate reactogenicity and systemic safety in both healthy and HIV infected adults (Durier and others 2006).

Vector-Based Vaccines

Vector-based vaccines have been explored as viable agents at eliciting anti-HIV specific immune responses. Using viral vectors as the vehicle to transport encoded genes for HIV protein regulatory agents, Gag p24, and envelop proteins, clinical trials have demonstrated the ability to induce HIV-specific T cells responses (Currier and others 2010; Peters and others 2007; Wilson and others 2008). However, T cell studies in patients with early HIV infection and LTNP revealed that Gag p24-specific T cells

possess greater function than Env-specific T cells at offering protection (Rosenberg and others 1997). Thus, Gag p24 proteins were highly used in the development of constructs. One of the largest recent vector-based vaccine clinical trials, The Step Study, used an Ad5 vector that encoded HIV Gag, Nef, and Pol genes. The goal was to elicit a cell-mediated immune response to control HIV once infected. However, the study was halted when the vaccinated group had more HIV infections than the placebo group and there was no effect on early HIV levels (Buchbinder and others 2008). Even though this was a failed attempt, the data generated will give us a better understanding of the immune response to HIV infection.

Dendritic Cells

History and Background of DCs

Paul Langerhans was the first to characterize DCs in 1868 when he mistakenly thought them to be nerve cells because of their long dendrite-like cytoplasmic extensions. A little over a century later, in 1973, Ralph Steinman and Zanvil Cohn were the first to fashion the term "dendritic cell". These cells were found to be the cornerstone of an immune response and potent stimulators of B and T lymphocytes.

There are different types of DCs naturally found in the human body that originate from two pathways. The myeloid DC (mDC) and plasmacytoid DC (pDC) are the result of the two pathways. mDC and pDC are both found circulating in the blood. However, only mDCs are found in the peripheral and secondary lymphoid tissue (Ueno and others 2007). In an immature state, the DC is in surveillance mode, sampling its surroundings by pinocytosis and phagocytosis and is equipped with an assortment of surface molecules

to mediate endocytosis (i.e., TLRs, macrophage mannose receptor, DEC-205, Fcγ and Fcε) (Steinman and Banchereau 2007).

Upon the detection of a foreign antigen, the immature DC differentiates from an antigen capturer to an antigen presenting cell. This, in turn, initiates migration via the efferent lymphatics to the T cell areas of secondary lymphoid organs. During this time, the DC changes its morphology, down-regulates endocytic/phagocytic receptors, secretes chemokines to attract specific cells needed for the response (i.e., CCL19, CCL21, and CCL22) (Dubsky and others 2005; Ueno and others 2007), secretes cytokines that differentiate and polarize the attracted cells, and up-regulates co-stimulatory molecules (i.e., CD40, CD80, and CD86) (Ueno and others 2007). Then, the DCs induce proliferation and differentiation of antigen-specific T cells (Rozis and others 2008).

Monocyte Derived DC

The ability of DCs to induce CTL and helper T cell responses are essential in the process of vaccination (Alvarez and others 2008; Banchereau and Steinman 1998; Sabado and Bhardwaj 2010; Steinman and Banchereau 2007; Yu and others 2008). Several groups have demonstrated the efficacy of DC vaccines in the therapeutic treatment of cancer and viral infections, including HIV (Banchereau and others 2005; Melief 2008; Palucka and others 2007; Timmerman and Levy 2000). Monocytes have been used as precursors to generate DC vaccines ex vivo. Various combinations of cytokines have been used to differentiate monocytes to DCs such as GM-CSF in combination with IL-4, IL-15, or IFN-α (Sallusto and Lanzavecchia 1994; Steinman and Banchereau 2007). They have been shown to elicit potent T cell responses in vitro (Ueno and others 2007) and in vivo (Palucka and others 2007) and thus have been employed as

vaccines in clinical trials to treat cancer and infectious disease (Connolly and others 2008; Palucka and others 2007).

There have been a number of DC vaccines developed here at Baylor Institute for Immunology Research (BIIR) and either have been or are currently being tested in phase I/II clinical trials. The most current vaccines are generated by culturing monocytes with granulocyte macrophage-colony stimulating factor (GM-CSF) in combination with IFN-α or IL-15. The cultured monocytes are pulsed with antigen(s) (e.g., killed melanoma cancer cells, antigen-specific peptides, and/or antigen fusion proteins) and activated (e.g., lipopolysaccharide (LPS) via TLR4, and/or CD40 ligand) for enhanced up-regulation of MHC I and II as well as co-stimulatory molecules responsible for T cell cross-talk.

IFN-α, β, and ω Type I cytokines exist naturally and are involved with the innate immune responses upon viral infection. Though a surface receptor complex called IFN-α receptor (IFNAR), they are able to elicit cellular responses. IFNAR is composed of two chains, IFNAR1 and IFNAR2. With the engagement of IFN/IFNR, Janus kinase (JAK) and signal transducer and activation of transcription (STAT) complex pathways are activated. The phosphoration of JAK/STAT pathways results in the downstream activation of gene transcription (Platanias 2005).

The pathogenic role of IFN-α in autoimmune disease, systemic lupus erythematosus (SLE), has been well documented (Dall'era and others 2005; Feng and others 2006; Kirou and others 2005; Niewold and others 2007). Studies have shown that an overabundance of IFN-α contained in SLE patient's serum is able to differentiate healthy donor monocytes into potent antigen presenting cells. IFN-α has been exploited

in DC development studies for the treatments of cancer and chronic viral infections (Bennett and others 2003; Blanco and others 2001).

IL-15 is pro-inflammatory cytokine that functions similarly to IL-2. It was first identified because of its ability to stimulate $CD8^+$ T cells (Steel and others 2010). It is a pleiotropic cytokine that signals via a heterotrimeric receptor that is shared with IL-2. IL-15 functions by *trans*-presentation through IL-2 β and γ chains as well as IL-15Rα that is present on DCs (Steel and others 2010).

The pathogenic role of IL-15 has been observed and documented in rheumatoid arthritis (RA) as well as a host of other inflammatory diseases (Waldmann 2006). Thus, scientists explored the use of IL-15 in the development of immunotherapies. Monocyte-derived DCs generated using GM-CSF and IL-15 have been observed to express Langerin (also known as CD207); a surface protein characteristically associated with Langerhans cells. When compared to GM-CSF/IL-4 DCs, GM-CSF/IL-15 DCs possess the ability to efficiently prime naïve $CD8^+$ T cells against melanoma antigens, and increases expression of IFN-γ on $CD8^+$ T cells (Dubsky and others 2007; Mohamadzadeh and others 2001). It has been shown that IL-15 cytokine alone reduces in vitro CD95/Fas-induced apoptosis of HIV-specific $CD8^+$ T cells (Mueller and others 2003) and blocking IL-15 in RA patients reduced the proliferation, induced apoptosis, and suppressed the release of IFN-γ by cells (Baslund and others 2005). However, this process lacks the ability to induce long lasting T cell memory responses.

TLRs function as the bridge between innate and adaptive immune responses which makes their ligands perfect adjuvants for vaccine development. DCs are equipped with surface TLRs expressed TLR1, 2, 4, and 6 and intracellular endosomal TLRs such

as TLR3, 7, 8, and 9. The surface TLRs recognize lipid structures and flagellin, whereas the endosomal TLRs recognize nucleic acids. Each of these TLR molecules, once signaled, elicits a distinct maturation signature of DCs (Ueno and others 2007).

In our studies, we focus on TLR2 and TLR4 activation, both of which are expressed on the surface of DCs. Literature suggests that lipopeptides composed of a palmitoyl-lipidated tail are highly efficient at inducing immune responses. Studies have demonstrated that the palmitoyl-lipidated peptides were able to activate immature DCs by up-regulating MHC I and II molecules as well as induce the secretion of IL-12 (Zhu and others 2004). Furthermore, they confirmed that the palmitoyl-lipidated peptides indeed exploit TLR2 signaling pathway on DC to elicit antigen-specific T cell

LPS is a cell wall component of gram negative bacteria. Historically, it has been observed to be the cause of systemic toxic shock syndrome. LPS signals through TLR4 and has been used as an adjuvant in ex vivo monocyte-derived DC studies for the potent activation affects. Studies have shown that signaling via TLR4 on monocyte-derived DCs using LPS induces up-regulation of MHC I and II molecules, as well as co-stimulatory molecules (CD80 and CD86), and induces Th1 T cells responses by the production of IL-12 (Ueno and others 2007).

Monocyte Derived DC Vaccines in HIV

DCs generated in the presence of GM-CSF and IFN-α (from monocytes of HIV patients) have been shown to highly stimulate $CD8^+$ T cell growth in vitro (Carbonneil and others 2003). It has also been shown that these DCs induce T cells that concomitantly produce type 1 (IFN-γ) and type 2 (IL-4 and IL-10) cytokines in vitro (Della Bella and others 2004). Additionally, Belardelli's group has shown inhibition of

viral infection when human PBL-SCID mice are vaccinated with IFN-α-generated DCs pulsed with aldithiol-2-inactivated HIV followed by challenge with HIV (Lapenta and others 2003).

IL-15 DCs have not yet been tested in vivo. However, owing to their Langerhans cell-type properties, these DCs are attractive for use in human clinical trials. In Dubsky's paper (Dubsky and others 2007), IL-15 DCs made from healthy donors have the ability in vitro to induce naïve $CD8^+$ T cells to polarize towards effectors that secrete type I cytokines, prime antigen-specific $CD8^+$ T cells, and up-regulate highly potent antigen-specific CTLs. These DCs also express cytokines (including membrane-bound IL-15 in active form; (Dubsky and others 2007) that help $CD8^+$ T cell priming and differentiation.

Aims of Dissertation

The aim of my dissertation is to develop monocyte-derived DC from HIV infected patients that are able to induce T cell responses similar to that of LTNP in vitro and that can be tested in proof-of-principle clinical trials. Vaccinating HIV patients with DCs to induce polyfunctional T cells that offer protection against HIV infected cells would negate the use of HAART and eliminate the toxic side effects and financial burden that the majority of patients are currently dealing with today. The specific aims of this dissertation are as follows:

1. Development of DALIA-1 vaccine: GM-CSF/IFN-α DCs vaccine pulsed with LIPO5 and activated with LPS.
2. Dendritic cells and lipopeptides immunizations for AIDS – DALIA-1 clinical trial.

3. Development of DALIA-2 vaccine: GM-CSF/IL-15 DCs vaccine pulsed with LIPO5 and activated with LPS.

Utilizing the ability to program immune responses through active immunotherapy, the studies presented in this dissertation provide evidence that DC vaccination of patients can elicit anti-HIV T cell responses and potentially offer protection further providing a window for the discontinuation of HAART.

CHAPTER TWO

Materials and Methods

Patient Population

For the development of GM-CSF/IFN-α DC vaccines, leukapheresis was collected from eight HIV-1-infected adult patients who were on the HAART regimen, identified as A1-A8, and eight healthy, non-HIV infected adult volunteers, identified as H1-H8 (Table 1) between October and December 2007 at the Baylor University Medical Center-Apheresis Collection Center (Dallas, TX). The HIV-1 patients who volunteered for this study were selected according to the following criteria: plasma HIV-1 RNA viral load level <50 copies/mL and $CD4^+$ T cell counts >500 cells/mL of peripheral blood. The HIV-1-infected patients were recruited at North Texas Infectious Disease Consultants in Dallas, TX. The study protocol 007-014 was approved by the Institutional Review Board of the Baylor Health Care System (Dallas, TX) and informed consent was obtained from all individuals participating in the study.

For the development of GM-CSF/IL-15 DCs, leukapheresis was collected from eight HIV patients on HAART between February 2009 and June 2009 at the Baylor University Medical Center-Apheresis Collection Center (Dallas, TX). The patients are identified as D1-1-D1-8. The HIV patients who volunteered for this study were selected according to the following criteria: plasma HIV-1 RNA viral load level <50 copies/mL and $CD4^+$ T cell counts >500 cells/mL of peripheral blood. The demographic, viral load and CD4 T cell counts are captured in Table 2. The HIV-1-infected patients were

recruited at the North Texas Infectious Disease Consultants in Dallas, TX. The study was approved by the Institutional Review Board of the Baylor Health Care System (Dallas, TX) and informed consent was obtained from all individuals participating in the study.

Table 1. Demographics of the patients enrolled in the study for the pre-clinical development of GM-CSF/IFN-α DC.

Healthy Donors			HIV-1 Infected Patients		
Donor Number	Age	Gender	Patient Number	Age	Gender
H1	24	Female	A1	44	Male
H2	34	Male	A2	40	Male
H3	30	Male	A3	35	Male
H4	50	Female	A4	39	Male
H5	43	Male	A5	45	Male
H6	23	Female	A6	47	Male
H7	28	Female	A7	35	Male
H8	27	Male	A8	46	Male

Generation of DC Vaccines

Monocyte Enrichment

Peripheral blood monocytes were enriched from the leukapheresis according to cellular density and size by elutriation (Elutra™, CaridianBCT, Lakewood, CO) as per the manufacturer's recommendations. The Elutra's automated program separated the cells into five fractions using various flow rates and centrifuge speed. Elutriation Fraction 5 consisted mainly of monocytes, ~85% on average, with the remainder of the cells being granulocytes, lymphocytes, eosinophils and basophils as measured by diagnostic hemacytometry ABX Pentra 60C+ (Horiba ABX Diagnostics, Montpellier, France), which incorporates cytochemistry, focused flow impedance, light absorbance

and flow cytometry for calculating complete blood cell count with differential. Cell counts and purity of the elutriated fractions were also assessed by ABX Pentra 60C+. The Fraction 5 of elutriated cells was used as the source of monocytes for production of the DC vaccine.

Table 2. Characteristics of the patients enrolled in the study for the pre-clinical development of GM-CSF/IL-15 DC.

HIV-1 Infected Patients				
Patient Number	Age	Gender	Viral Load (copies/mL)	$CD4^+$ T cell counts (cells/μL)
D1-1	37	Male	<48	577
D1-2	52	Male	<48	646
D1-3	51	Male	<48	679
D1-4	31	Male	<48	898
D1-5	49	Male	<48	1430
D1-6	32	Male	<48	1218
D1-7	46	Male	<48	682
D1-8	46	Male	<48	720

Dendritic Cells Production-GM-CSF/IFNα DC

On Day 0 of the process, the elutriated monocytes were resuspended in serum-free CellGro® DC culture media (CellGenix Technologie Transfer GmbH, Germany) at a concentration of $1x10^6$ cells/mL for culture in disposable plastic culture bags (AFC, Gaithersburg, MD). The media was supplemented with 100 ng/mL GM-CSF (Leukine®, Berlex, Wayne, NJ) and 500 IU/mL IFN-α (Intron A®, IFN-α-2b, Merck/Schering-Plough, Kenilworth, NJ). After 24 hours in culture, fresh cytokines were added. On day 2, LIPO-5 was added at various concentrations, over a range of 0.003 μM to 0.3 μM per peptide. On Day 3 of culture, 6 hours prior to cell harvest, LPS (NIH, Bethesda, MD)

was added to the cell suspension at 5 EU/mL to activate the LIPO-5-loaded DC. After LPS activation, the DC were harvested, washed with normal saline (0.9% NaCl, USP grade; Hospira, Lake Forest, IL), and suspended at $30x10^6$ viable cells/mL in freezing-solution for filling into glass vaccine vials. The DC vaccine freezing-solution consisted of 80% heat-inactivated autologous serum, 10% Plasma-Lyte A (Hospira) supplemented with 5% dextrose (Baxter, Deerfield, IL) and 10% dimethyl sulfoxide (DMSO) (Cryoserv®, Bioniche, Lake Forest, IL). The cells were frozen in a rate-controlled freezer and the vials were stored at -180ºC in the vapor-phase of a liquid nitrogen tank.

The DC vaccines were thawed and diluted 10-fold with normal saline. For culture, cells were centrifuged and resuspended in cRPMI medium (RPMI 1640 with 2.5% hepes, 1% penicillin/streptomycin, 1% non-essential amino acids, 1% L-glutamine, and 0.1% β-mercaptoethanol (Invitrogen, Carlsbad, CA) supplemented with 10% human serum type AB (Gemini Bio-Products, West Sacramento, CA, US)).

Dendritic Cells Production-GM-CSF/IL-15 DC

Monocytes that were enriched from the patient's apheresis blood product by elutriation and frozen at $50x10^6$ viable cell/mL per vial. On Day 0 of the DC vaccine culture, the monocytes were thawed, washed and suspended at a concentration of $1x10^6$ cells per mL in CellGro® DC culture media (CellGenix Technologie Transfer GmbH, Germany) supplemented with 100 ng/mL of GM-CSF (Leukine®, Berlex, Wayne, NJ) and 100 ng/mL of IL-15 (CellGenix Technologie Transfer GmbH, Germany). DC vaccines were generated in 50 mL cell culture bags (AFC, Gaithersburg, MD). After 24 hours of culture, fresh GM-CFS and IL-15 was added to the media in each cell culture bag. On day 3, after ~72 hours of cell culture, the cells in the culture bags are pulsed

with 0.1 μM of LIPO-5 to load the DC with HIV antigens. On Day 4 of culture, 6 hours prior to cell harvest, lipopolysaccharide (LPS; NIH, Bethesda, MD) was added to the cell suspension at 5 EU/mL to activate the LIPO-5-loaded DC. After LPS activation, the DC were harvested, washed, and suspended for culture in cRPMI medium at $1x10^6$ viable cells/mL.

Peptides for Loading DC

The ANRS-HIV LIPO-5 vaccine is a mixture of five HIV-1-antigen lipopeptides. Each peptide was modified in the C-terminal position with an N-ε-palmitoyl-lysylamide group. The LIPO-5 vaccine, supplied as a lyophilized mixture of 500 μg of each lipopeptide (2.5 mg of lipopeptide/vial), was dissolved in 5% dextrose (USP grade, Hospira) injectable solution to a stock concentration of approximately 134 μM per lipopeptide. The five ANRS-HIV-1 LIPO-5 antigen peptides are:

Gag_{17-35} (EKIRLRPGGKKKYKLKHIV K(Palm)-NH_2)

$Gag_{253-284}$ (NPPIPVGEIYKRWIILGLNKIVRMYSPTSILD K(Palm)-NH_2)

Nef_{66-97} (VGFPVTPQVPLRPMTYKAAVDLSHFLKEKGGL K(Palm)-NH_2)

$Nef_{116-145}$ (HTQGYFPDWQNYTPGPGVRYPLTFGWLYKL K(Palm)-NH_2)

$Pol_{325-355}$ (AIFQSSMTKILEPFRKQNPDIVIYQYMDDLY K(Palm)-NH_2).

The same HIV-1-antigen peptides (that is, peptides with the identical sequence to those in LIPO-5, but without the palmitoyl-lysylamide moiety; 'non-lipidated peptides') were used in the autologous T cell response assay. The stock concentration of the non-lipidated peptides was 10 mM per peptide suspended in DMSO.

Gag_{17-35} (EKIRLRPGGKKKYKLKHIV)

$Gag_{253-284}$ (NPPIPVGEIYKRWIILGLNKIVRMYSPTSILD)

Nef_{66-97} (VGFPVTPQVPLRPMTYKAAVDLSHFLKEKGGL)

$Nef_{116-145}$ (HTQGYFPDWQNYTPGPGVRYPLTFGWLYKL)

$Pol_{325-355}$ (AIFQSSMTKILEPFRKQNPDIVIYQYMDDLY)

Overlapping 11 amino acid (AA) peptide libraries (Bio Synthesis, Lewisville, TX) of the HIV-1 Gag and Nef proteins were used to define the HIV-1-antigen epitopes. The peptide library stock concentration was 10 mM per peptide in an acetonitrile (Fluka, Buchs, Germany) solution.

Cell Count, Viability, and Recovery

Using trypan blue (Invitrogen) reagent, a hemacytometer and light microscopy percentage of viable cells and total cell counts were calculated. Cell counts were determined by diluting an aliquot of cells with trypan blue at a ratio of 1:1. Observing the cells in a microscope, viable cells are considered white and non-viable cells are considered non-viable. The non-viable cells are blue due to the uptake of trypan blue.

Total cells were calculated by multipling the average of total viable cells counted on three 16-square grid of a hemacytometer with the dilution, microscope magnification, and total volume of media cells are suspended in with which the aliquot sampled. Viability of cells were calculated by dividing the viable cells by the sum of viable and non-viable cells then multiplying by 100 for percentage of viable cells. Recovery was calculated by dividing the total number of cells counted by trypan blue divided by the number of cells frozen per vial then multiplied by 100 for percentage of recovered cells after freezing.

Characterization of Monocytes and DC Vaccines

Phenotype of Monocytes

Antibodies used to stain monocytes were CD86, CD40, CD11b, HLA-DR, CD14, CD45 (all from BD Bioscience), CD16 (Caltag), and CD62L (IOTest). Cells were acquired by LSRII (BD Bioscience) and analyzed with FlowJo software (Treestar, San Carlos, CA).

Phenotype of DC Vaccines

The phenotypes of the DC vaccines were assessed using fluorescence-conjugated monoclonal antibodies: CD14-PE, HLA-DR-PE, CD11c-APC, CD80-PE, CD83-PE, CD207-PE (IO Test & Validation, Bailey, Roxburghshire, UK), and CD1b/c-FITC (Biosource, Carlsbad, CA). Cell staining profiles were acquired on a FACSCalibur™ Flow Cytometer (BD Bioscience, San Jose, CA) and analyzed with FlowJo software (Treestar, San Carlos, CA).

Morphology of DC by Giemsa Staining

The DC morphology was observed by Giemsa staining. In brief, DCs are centrifuged by a Shandon cytospin (Thermo Shandon, Pittsburgh, PA), to adhere to a glass slide. The slide is then treated with Dif-Quick fixative, solution I, solution II (all from Siemans, IL), and rinsed. Once the glass slide was dry, a cover slip was fixed over the cells. Examination of the Giemsa stained cells was visualized by an Olympus BX60 light microscope with 40x and 100x magnification objective lens and images were captured with a Nikon Digital Camera (DXM1200C) and Nikon NIS Elements Software (Nikon, CA).

DC Cytokine and Chemokine Secretion Assay

The cytokine and chemokine secretion profile of the DC vaccine was assessed by culturing 200,000 thawed DC suspended in cRPMI medium in 96-well microtiter plates. rhCD40L (R&D Systems, Minneapolis, MN) at 100 ng/mL was added to select wells. The supernatant was harvested after 48 hours and frozen. UV-irradiation was performed on the thawed supernatants to inactivate any HIV virion contaminates. Supernatants were then analysed by Luminex® cytokine multiplex bead-based platform using Bio Plex 200 and Bio Plex Manager 5.0 software (Bio Rad, CA). The following cytokines and chemokines were analyzed: IL-1β, IL-6, IL-8, IL-10, IL-12p40, IL-12p70, IP-10, MCP-1, MIP-1α, RANTES, and TNF-α.

HIV Viral Load

Detection of HIV viral replication in the DC vaccine was measured by HIV p24 Antigen ELISA. In brief, the HIV p24 Antigen ELISA was performed using a 96 flat bottom plate coated with a monoclonal antibody to HIV p24. For quantitative analysis, the positive control was diluted with media (or cell culture) in 2 fold dilutions starting at 100 pg/mL. For each positive control dilution, 200 µL were plated including media (or cell culture) and blank for negative controls. The samples were plated at a volume of 200 µL. All conditions were tested in duplicates. To each of the samples, 20 µL of Triton-X100 was added to lyse the cells. The plate was incubated for 2 hours at 37° then washed 6 times with wash buffer. Detector antibody was added at 100 µL to each well and the plate was incubated for 1 hour at 37° then washed. Streptavidin-HRP diluted 1:100 and 100 µL was added to each well. The plate was incubated for 30 minutes at room temperature then washed. OPD Substate solution was made and 100 µL was added

to each well. The plate was incubated for 30 minutes at room temperature in the dark. Stop solution was added at 100 μL to each of the wells and read on a spectrophotometer using 490 nm wavelength. If the sample contains HIV p24 from the virus, the antibody coated on the plate will capture the protein and will be detected by the antibody and seen as a color change by streptavidin-HRP and OPD Substrate. The stop solution allowed the reaction to be stopped and read.

ANRS LIPO-5 consists of five HIV peptides, that is, Gag p17(17-35), Gag p24(253-284), Nef(66-97), Nef(166-145), and Pol(325-355). Among these peptides is a peptide from Gag p24 in which the HIV p24 antigen ELISA kit (Perkin Elmer, Waltham, MA) is used to detect the viral load. To assure the Gag p24 (253-284) would not interfere with the study, an ELISA Test was performed using culture media and the Gag p24 (253-284) at the concentration used to load the vaccine and tenfold higher. Once the results confirm that the peptide would not interfere, DC studies were pursued.

The samples obtained for analysis of viral load, measured by HIV p24 Antigen ELISA, were $1x10^6$/mL cells suspended in culture supernatant retained after 4 hours, 20 hours, and 72 hours which is the time of harvest. Two DC vaccines were manufactured from frozen monocytes. One DC vaccine was of healthy uninfected donor H1 and one DC vaccine was of HIV donor A5. The DC vaccines were pulsed with ANRS LIPO-5 at a concentration of 0.1 μM per peptide. In order to measure the total viral load, supernatant and cells were used in one sample. To obtain lysates, some samples were snap-frozen with liquid nitrogen and thawed three times followed by centrifugation to remove debris. Other samples were used untouched in the ELISA in which the cells are lysed by Triton-X100.

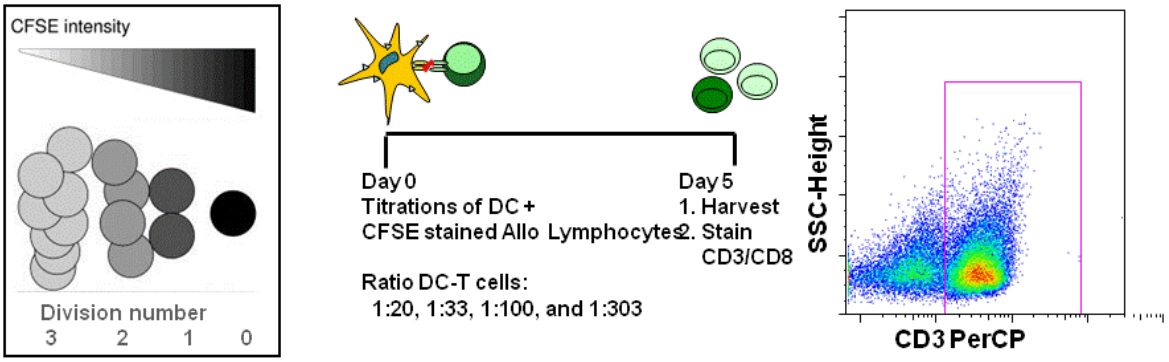
DC Vaccines and T Cell Co-Cultures

Allogeneic-MLR

The potency of the DC vaccine was assessed by using an allogeneic T cell proliferation assay (Scheme 1). Lymphocytes were isolated by elutriation from apheresis blood product of a healthy, non-HIV-1-infected patient and frozen. For the assay, lymphocytes were thawed, washed by centrifugation, stained with carboxyfluorescein succinimidyl ester (CFSE, Invitrogen), and resuspended at a concentration of $1x10^6$ cells/mL in cRPMI medium. Using a 96-well plate, DC and lymphocytes were added to designated wells for a final ratio of 1:20, 1:100, and 1:500, that is, 5000, 1000 and 200 DC to 10^5 lymphocytes, respectively. The negative (C-) and positive (C+) controls for this assay were lymphocytes cultured in media alone or with CD3/CD28 Dynabeads (Invitrogen), respectively. Each control and test condition was cultured in triplicate. After five days of co-culture, the cells were harvested from the 96-well plate, stained with anti-CD3 and anti-CD8 fluorescence-conjugated monoclonal antibodies (CD3-PerCP and CD8-APC from BD Bioscience) and analyzed by flow cytometry to determine the percentage of dividing $CD3^+CD8^+$ T cells and $CD3^+CD8^-$ T cells ($CD4^+$ T cells) based on reduced CFSE staining intensity.

Autologous Lymphocyte Reaction-Lymphocytes

Lymphocytes enriched in Elutriation Fractions 2 and 3, and stored frozen, were isolated from the same HIV-1 patient's leukapheresis that was used to produce the DC vaccine from Elutriation Fraction 5. The frozen lymphocytes were thawed, washed by centrifugation, and then resuspended at a concentration of $1x10^6$ cells/mL in cRPMI



Scheme 1. Culturing protocol for measuring potency of DC to elicit allogeneic lymphocyte proliferation by CFSE. Allogeneic cells are stained with CFSE and cultured with DC on Day 0. Day 5 the expanded-allogeneic T cells are harvested and stained with fluorescence-conjugated monoclonal antibodies against CD3, CD8 and CD4. The cells are acquired by FCM and analyzed by FlowJo software. The analysis by FlowJo software begins by gating the typical population for lymphocytes according to forward scatter (FSC) and side scatter (SSC). The second gate selects $CD3^+$ cells for further analysis.

medium. Autologous DC vaccine and lymphocytes were co-cultured, in duplicate or triplicate, in a 24-well tissue culture plate at a ratio of 1:20 (100,000 DC to $2x10^6$ lymphocytes) or 1:200 (10,000 DC to $2x10^6$ lymphocytes) and incubated for a total of 10 days. Ten IU/mL IL-7 (R&D Systems) was added to cultures. On day 2, 100 IU/mL IL-2 (Bayer Healthcare, Emeryville, CA) was added.

Intracellular Cytokine Staining

DC-vaccine-expanded lymphocytes were harvested at day 10 and were restimulated with individual non-lipidated HIV-1-antigen peptides for 4 hours. Each DC-vaccine-stimulated lymphocyte condition was restimulated without peptides (background control, C-) or with PMA 2 ng/mL and Ionomycin 1 µM (PMA/IONO) as an IFN-γ-positive control (C+). After one hour of restimulation, BD Golgistop™ was added to

block any further protein transport. After a total of four hours, the cells were harvested and cell surface (CD3 and CD8) and intracellular (IFN-γ) staining was performed.

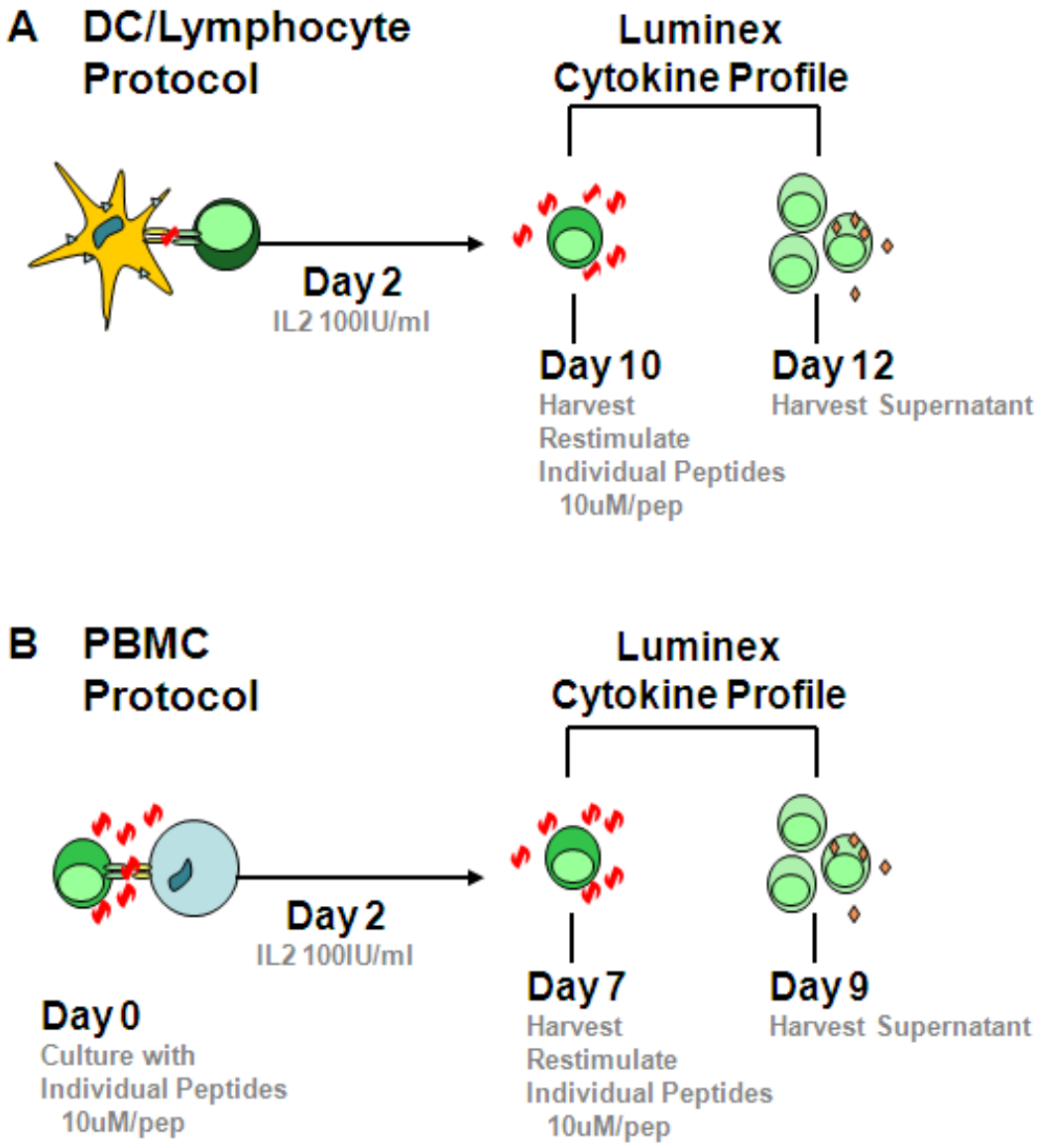
Restimulated lymphocytes were surface stained with anti-CD3 and anti-CD8 fluorescence-conjugated monoclonal antibodies as well as a cell viability dye, Aqua LIVE/DEAD. The cells were then washed, permeabilized with BD permeablization/fixation solution, and stained with fluorescence-conjugated intracellular cytokines anti-IFN-γ, anti-perforin, anti-granzyme A and B monoclonal antibodies. The cells were then fixed with 1% paraformaldehyde, acquired by FACS, and analyzed by FlowJo software.

Cytokine Secretion

DC-vaccine-expanded lymphocytes were harvested at day 10 and were restimulated with individual non-lipidated HIV-1-antigen peptides or individual 15 AA peptides for 48 hours. Each condition was restimulated without peptides (background control, C-). The supernatant was collected and analyzed by multiplex beads assay (Luminex) to detect IL-10, IL-13, IL-21, and IFN-γ.

Epitope Mapping

Autologous lymphocytes from HIV-1 patients A7 and A8 were cultured with LIPO-5-loaded DC vaccine for 10 days (Scheme 2A). As a control, PBMC collected from HIV-1 patients A7 and A8 were cultured for 7 days in media containing individual 15 AA peptides at 10 μM from HIV-1 proteins Gag and Nef peptide libraries (Scheme 2B). At day 2, 100 IU/mL IL-2 was added. At the end of the "antigen-priming" culture, the DC vaccine expanded lymphocytes and the peptide-library-stimulated PBMC were

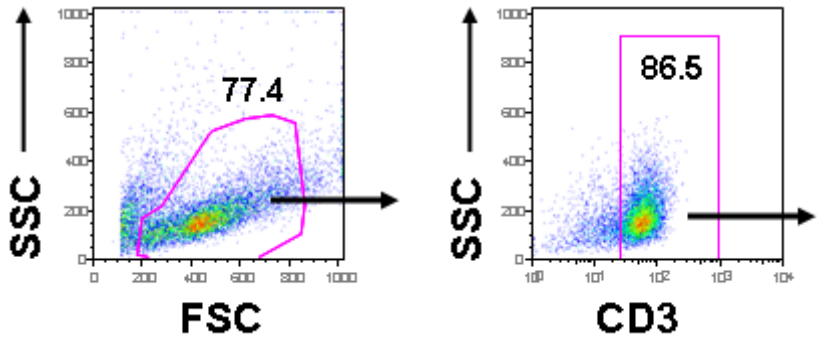


Scheme 2. Representative scheme of the culture protocol used to perform the in vitro vaccinations. (A) The protocol in which the LIPO-5-loaded DC vaccines are cultured with lymphocytes at a ratio of 1:20; day 2, IL-2 at 100 IU/mL is added; day 10, cells are harvested and restimulated using individual 15mer peptide libraries of Gag and Nef at 10 μM/peptide for 48 hours followed by supernatant harvest and analysis by Luminex. (B) The protocol in which the PBMC are cultured with individual 15mer peptides of HIV Gag and Nef peptide libraries at a concentration of 10 μM/peptide; day 2, IL-2 at 100 IU/mL is added; day 7, cells are harvested and restimulated using the original peptide at 10 μM/peptide for 48 hours followed by supernatant harvest and analysis by Luminex.

harvested, washed and dispensed into the wells of a 96-well microtiter plate. To each well, one of the peptides from the HIV Gag and Nef peptide libraries was added to the cell culture media at 10 μM. The cells in each well were restimulated with one of the individual 15 AA HIV-1-antigen peptides for 48 hours. Supernatants were collected and cytokine multiplex beads assays (Luminex) were employed to analyze IFN-γ, IL-10, and IL-13.

FlowJo Analysis

Cells were analyzed by FlowJo software using gating strategies as in Scheme 3 to determine percentage of positive cells bearing fluorescence-conjugated antibodies on the surface or intracellular. Mean fluorescence intensity was measured on populations to determine the difference in staining.



Scheme 3. Gating strategy used to analyze intracellular IFN-γ staining by FACS. The first gate selects the typical population for lymphocytes according to forward scatter (FSC) and side scatter (SSC). The second gate selects $CD3^+$ cells for further analysis.

CHAPTER THREE

Preclinical Development of Therapeutic DC for HIV-Infected Patients – GM-CSF/IFN-α DC

Rationale

In preparation for a clinical trial to vaccinate HIV-infected patients on HAART with monocyte-derived DCs, we conducted a series of preclinical studies. DC vaccines were prepared by culturing elutriated enriched monocytes from HIV patients with GM-CSF and IFN-α. On day two, the cultured monocytes were loaded with either a mixture of five HIV antigen peptides or the same mixture of peptides that had been conjugated with a lipid tail (LIPO5 kindly provided by the National Agency of AIDS Research, ANRS). In the final 6 hours of culture, the antigen-loaded DCs were then activated with bacterial LPS and frozen. The DC vaccines were studied to determine the viability, quality, and potency. The functional potencies of the DC vaccines were quantified by their phenotype and by their ability to stimulate both an allogeneic MLR and an antigen-specific autologous T cell response.

Results

Monocyte Phenotype from HIV and Healthy Donors

As seen in Figure 1, the monocyte phenotype with regard to the number of $CD14^+HLA\text{-}DR^+$ cells and $CD14^+CD62L^+$ cells was similar between HIV patients and healthy uninfected donors. However, compared to healthy donors, a higher proportion of monocytes from HIV donors express the $CD14^+CD86^+$ (7.89% versus 1.93%),

$CD14^+CD40^+$ (7.34% versus 0.49%) and $CD14^+CD16^+$ (37.3% versus 17.5%) phenotype. HIV-1 infected patients have an increased population of peripheral $CD14^+CD16^+$ double positive monocytes which has been observed by several groups (Amirayan-Chevillard and others 2000; Pulliam and others 1997; Thieblemont and others 1995; Ziegler-Heitbrock 2007). The second objective of this study, having verified the process for isolating monocytes, was to determine whether monocytes from HIV donors could be used to generate a viable DC vaccine.

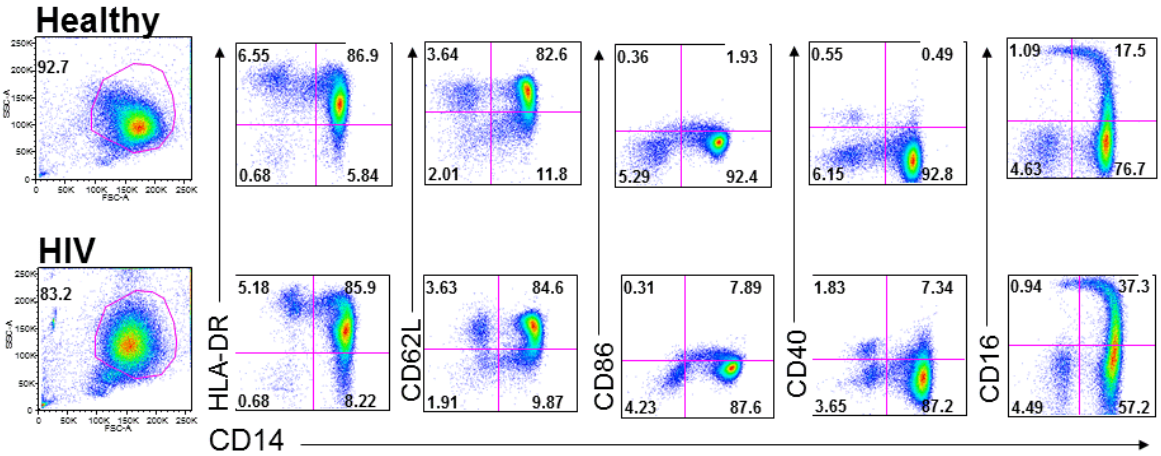


Figure 1. Phenotype of enriched monocytes collected in Fraction 5 from elutriated apheresis. A sample was taken directly from the collection bag and was stained with a variety of fluorochrome-conjugated antibodies. The cells were stained for 20 minutes at 4°C, the cells were washed, fixed with 1% paraformaldehyde and analyzed by FACS.

Comparison of Monocyte Enriched by Apheresis of HIV and Healthy Donors

The purity of the monocytes collected in elutriation Fraction 5 was comparable between HIV-1-infected patients (85.0 ± 8.4%) and healthy donors (87.7 ± 4.2%) (Table 3). Likewise, the percentage of elutriation Fraction 5 lymphocytes, neutrophils, eosinophils and basophils were similar between the two donor groups (Table 3). The total number of monocytes isolated from HIV patients (22.8 ± 9.3 $x10^8$) was higher but

not significantly different (T-test, $p < 0.079$) than that of healthy donors (15.3 ± 6.2 $x10^8$).

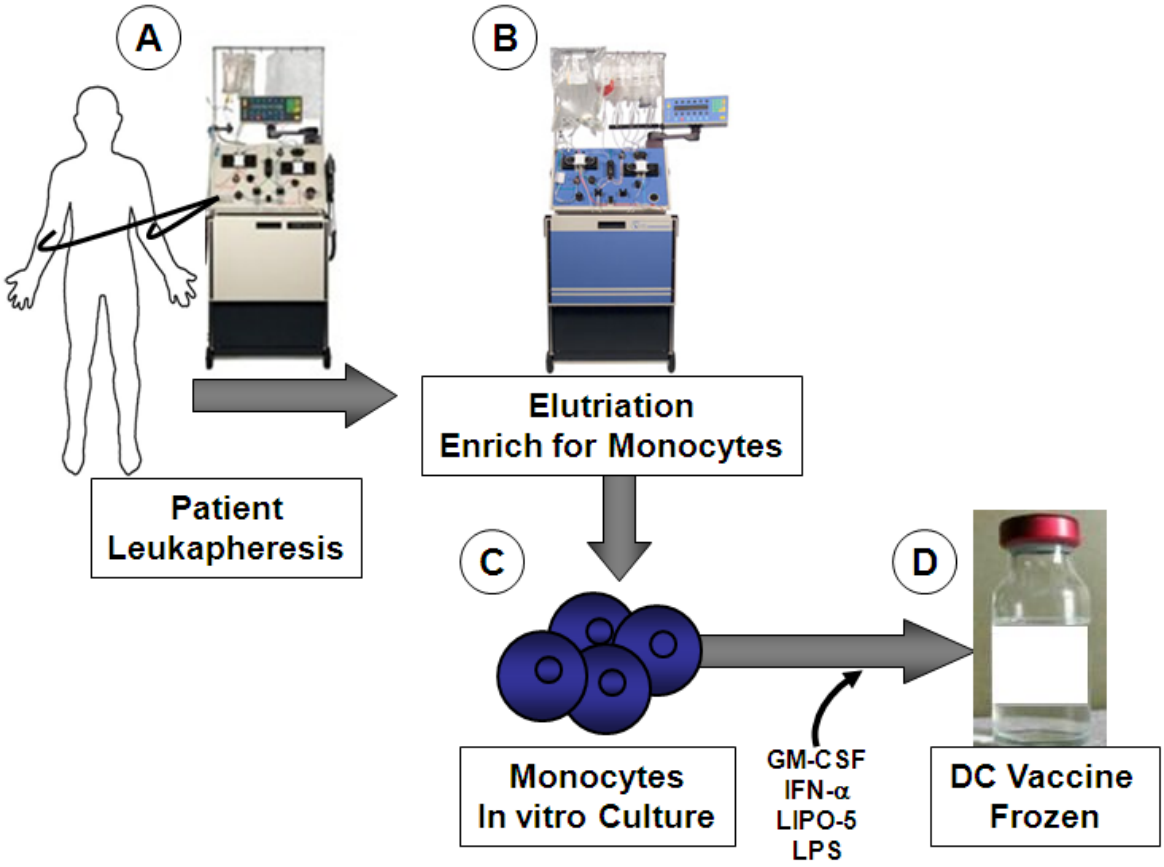
Table 3. Distribution of cells in elutriation fraction 5 collected from HIV-1-infected patients and healthy donors.

Cell Type	Healthy Donors	HIV-1 Infected Patients
Monocytes	87.7 ± 4.2*	85.0 ± 8.4
Neutrophils	2.8 ± 3.1	5.8 ± 10.3
Lymphocytes	8.3 ± 3.2	7.9 ± 3.7
Eosinophils	0.1 ± 0.2	0.2 ± 0.3
Basophils	1.1 ± 0.6	1.1 ± 0.3

*Data are presented as the mean ± standard deviation percentage of each cell type present in elutriation fraction 5 collected from the 8 healthy donors and 8 HIV-1-infected patients.

Generation of DC from HIV Monocytes

Antigen-loaded DC vaccines were manufactured in three-day monocyte culture with GM-CSF and IFN-α, as described in Material and Methods and illustrated in Scheme 4. Thawed DC vaccines were used to conduct the characterization assays described below. The phenotype of a typical DC vaccine manufactured with monocytes from an HIV-1-infected patient (A4) is shown in Figure 2 and results obtained with patients A1-A6 are summarized in Table 4. The DC vaccines had a high frequency of HLA-DR^+/$CD11c^+$ (mean, 94%; range, 88-98%) and $CD80^+$ cells (mean, 95%; range, 91-97%). Most DCs expressed CD1b/c^+ (mean, 62%; range, 36-86%), $CD14^+$ (mean, 62%; range, 17-87%), and $CD83^+$ (mean, 74%; range, 32-96%). A fraction of cells expressed $CD207^+$ (Langerin; mean, 28%; range, 7-46%). Thus, the DC vaccines express surface molecules characteristic of DC with co-stimulatory molecules necessary for T cell interaction.



Scheme 4. Schematic representation of GM-CSF/IFN-α DC vaccine manufacturing. (A) Collection of PBMC by leukapheresis. (B) Leukapheresis is separated by cellular density and size. (C) Enriched monocytes are cultured with GM-CSF and IFN-α, loaded with LIPO-5 (0.1 µM), activated with LPS, (D) harvested and frozen.

Characteristic of DC Morphology

Giemsa staining revealed the standard morphology of dendritic cells which is homogenous smooth intensely stained cytoplasm, cytoplasmic protrusions, a laterally positioned nucleus, and a ring of intense staining next to cell's plasma membrane (pictures not shown).

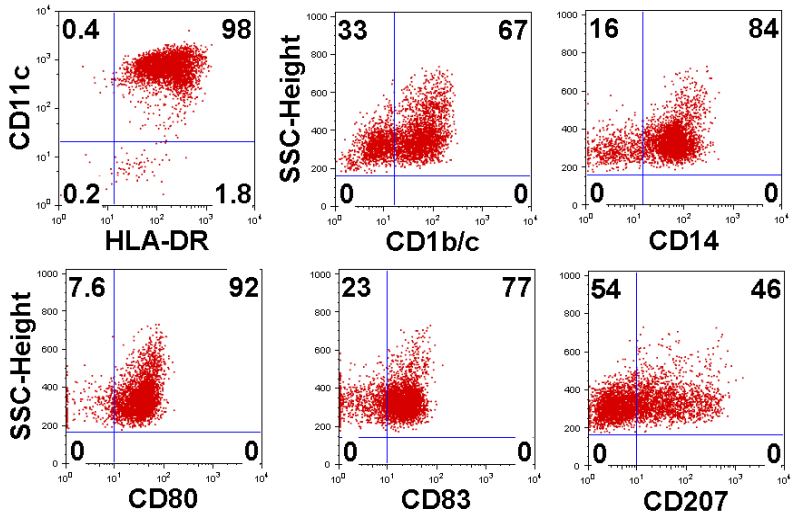


Figure 2. Representative DC phenotype by dot plot analysis of LIPO-5 loaded GM-CSF/IFN-α DC vaccine. FACS analysis dot-plots of the phenotype of ANRS HIV-LIPO-5-loaded DC vaccine from HIV-1 patient A4. DCs were thawed, washed, stained with various fluorescence-conjugated monoclonal antibodies, acquired on FACS Calibur, and analyzed by FlowJo Software.

Table 4. Summary of phenotypes of GM-CSF/IFN-α DC vaccines prepared with monocytes from HIV-1 infected patients A1-A6.

DC Vaccine	HLA-DR$^+$/CD11c$^+$	CD1b/c$^+$	CD14$^+$	CD80$^+$	CD83$^+$	CD207$^+$
A1	94*	60	17	96	95	19
A2	96	62	39	97	96	27
A3	88	36	67	96	77	7
A4	98	67	84	92	77	46
A5	98	86	76	96	32	42
A6	91	58	87	91	68	28
Mean	94.2	61.5	61.6	94.8	74.2	28.3
Std. Dev.	3.9	16	27.8	2.5	23.5	14.5

*Values represent the percentage of cells in the DC vaccine that express the specified cell surface marker(s).

DC Vaccines Secrete Pro-Inflammatory Cytokines and Chemokines

We analyzed the cytokines and chemokines secreted within 48 hours by HIV-DC vaccines either spontaneously (Table 5 and 6) or after CD40 ligation (Table 7 and 8). The

Table 5. LIPO-5 loaded GM-CSF/IFN-α DC vaccines from HIV patients secrete pro-inflammatory cytokines.

	Cytokines						
Patient	IL-1β	IL-6	IL-8	IL-10	IL-12p40	IL-12p70	TNF-α
A1	658*	3326	4951	38	1	18	20
A2	746	14936	4597	104	307	29	109
A3	3991	27978	5094	293	1510	165	626

*Data are presented as pg/mL.

DC vaccines generated from three patients secrete spontaneously high levels of pro-inflammatory cytokines, IL-1β (660-4000 pg/mL), IL-6 (3300-28000 pg/mL), and IL-8 (4600-5100 pg/mL). IL-10 was also secreted in low amounts ranging from 40-300 pg/mL. DCs from 2 of the 3 patients were found to secrete IL-12p40 (300-1500 pg/mL) and IL-12p70 (30-170 pg/mL). TNF-α was also secreted (20 to 630 pg/mL). The DCs secreted a panel of chemokines, including MCP-1 (CCL2, 480-5500 pg/mL), MIP-1α (CCL3, 3700-4500 pg/mL), RANTES (CCL5, 710-1120 pg/mL), and the interferon-inducible chemokine IP-10 (CXCL10, 1800-11400 pg/mL). In one analyzed patient, the addition of soluble rhCD40L to the DC vaccine culture resulted only in moderate if any enhancement of cytokine or chemokine secretion (Table 7 and 8). Thus, the DC vaccine demonstrates features of activated DCs.

DCs Culturing Conditions Does Not Support Replication of HIV

In brief, the HIV p24 Antigen ELISA was performed using a 96 flat bottom plate coated with a monoclonal antibody to HIV p24. For quantitative analysis, the positive control was diluted with media (or cell culture) in 2 fold dilutions starting at 100 pg/mL. For each positive control dilution, 200 μL were plated including media (or cell culture)

Table 6. LIPO-5 loaded GM-CSF/IFN-α DC vaccines from HIV patients secrete chemotactic chemokines.

	Chemokines			
Patient	MCP-1	MIP-1α	RANTES	IP-10
A1	481*	3703	712	1804
A2	1073	4500	1120	8974
A3	5497	4500	952	11420

*Data are presented as pg/mL.

Table 7. LIPO-5 loaded GM-CSF/IFN-α DC vaccines from HIV patient A3 secrete pro-inflammatory cytokines.

	Cytokines						
Patient/ Activation	IL-1β	IL-6	IL-8	IL-10	IL-12p40	IL-12p70	TNF-α
A3/None	3991*	27978	5094	293	1510	165	626
A3/rhCD40L	4760	33694	4750	408	2992	321	1365

*Data are presented as pg/mL.

Table 8. LIPO-5 loaded GM-CSF/IFN-α DC vaccines from HIV patient A3 secrete chemotactic chemokines.

	Chemokines			
Patient/ Activation	MCP-1	MIP-1α	RANTES	IP-10
A3/None	5497*	4500	952	11420
A3/rhCD40L	7370	4500	1149	10968

*Data are presented as pg/mL.

and blank for negative controls. The samples were plated at a volume of 200 μL. All conditions were tested in duplicates. To each of the samples, 20 μL of Triton-X100 was

added to lyse the cells. The plate was incubated for 2 hours at 37° then washed 6 times with wash buffer. Detector antibody was added at 100 µL to each well and the plate was incubated for 1 hour at 37° then washed. Streptavidin-HRP diluted 1:100 and 100 µL was added to each well. The plate was incubated for 30 minutes at room temperature then washed. OPD Substate solution was made and 100 µL was added to each well. The plate was incubated for 30 minutes at room temperature in the dark. Stop solution was added at 100 µL to each of the wells and read on a spectrophotometer using 490 nm wavelength. If the sample contains HIV p24 from the virus, the antibody coated on the plate will capture the protein and will be detected by the antibody and seen as a color change by streptavidin-HRP and OPD Substrate. The stop solution allowed the reaction to be stopped and read.

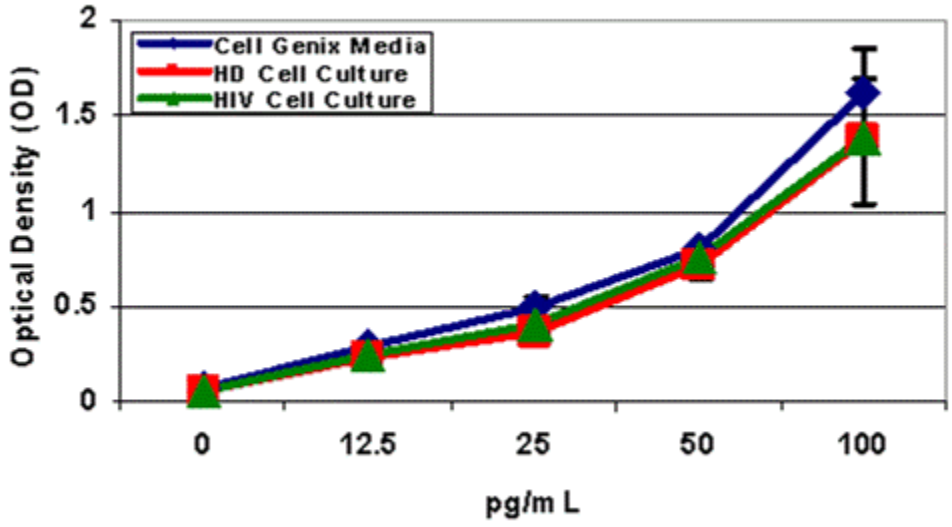


Figure 3. Analysis of titrated positive control using 72 hour cell culture supernatants for healthy donor H1 or HIV patient A5 cell culture or media alone.

Assessment of the non-LIPO-5 peptide HIV Gag p24 (253-284) interference in the ELISA, was concluded to be negative. This allowed further studies of HIV patient A5 and healthy donor H1 DC vaccines. DC vaccines aliquots that were harvested at 4

hours and 20 hours were negative for HIV p24 antigen. To ensure there were inhibitory factors of the culture, a sample of the 72 hour cell cultures were spiked with HIV Gag p24 positive control and titrated as done for the positive control with media. The comparison between the positive control titrated with media and positive control titrated with cell cultures are recorded in Figure 3. The samples at 72 hours were also negative for HIV p24 antigen.

DCs Elicit Potent Allogeneic Response

Allogeneic T cells collected from normal healthy donors were stained with CFSE. One hundred thousand lymphocytes were cultured with the DC vaccine added at various ratios from 1:20 to 1:500. After 5 days, the cells were stained with fluorochrome-conjugated anti-CD3 (PerCP) and anti-CD8 (APC) and analyzed by flow cytometry. Figure 4 represents a dot plot analysis of the MLR assay performed with the DC vaccine from HIV-1 patient A3. The cells are first gated on the $CD3^+$ T cell population and further analyzed by assessing CFSE staining intensity in the $CD8^+$ and $CD8^-$ T cells. Table 9 reports the allogeneic T cell responses of the total $CD3^+$ population for HIV-1 patient DC vaccines (A1-A6). At a ratio of 1:20, a mean of 43% of $CD3^+$ T cells dilute CFSE (range, 13-85%). At a ratio of 1:100, 20% of $CD3^+$ T cells diluted CFSE (range, 7-36%). At a ratio of 1:500, 4% of $CD3^+$ T cells diluted CFSE (range, 0.2-8%). The proliferation background (C-) was 0.9% (range, 0.1-1.6%). CD3/CD28 dynal beads were used as a positive control, and 72% of $CD3^+$ T cells diluted CFSE (range, 56-88%). Thus, monocyte-derived GM-CSF/IFN-α, ANRS HIV-LIPO-5 loaded, LPS-activated DC vaccines generated from HIV-1 patients act as potent antigen presenting cells in the allogeneic MLR.

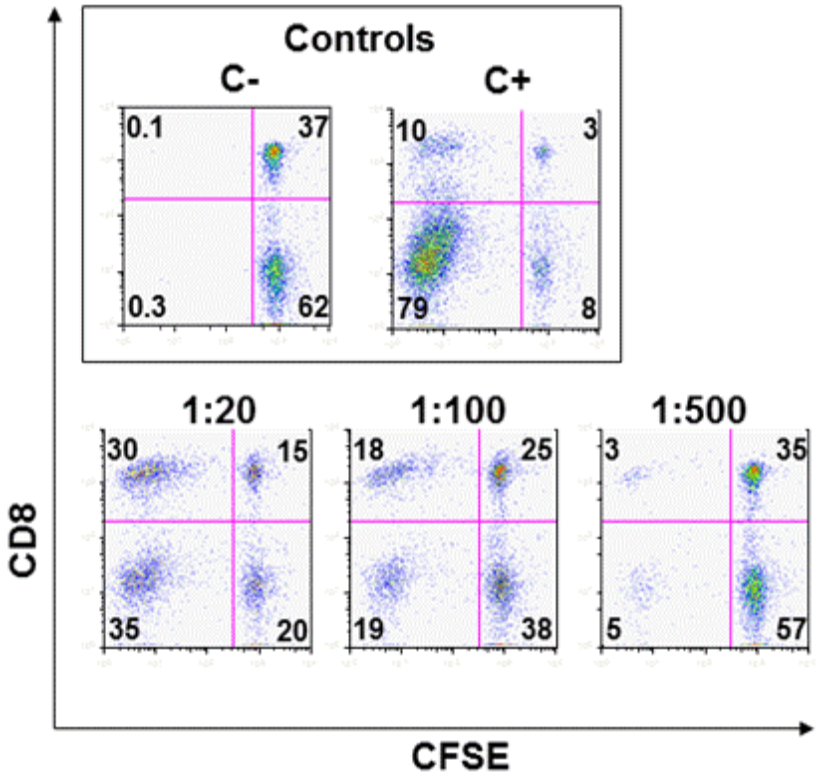


Figure 4. LIPO-5 loaded GM-CSF/IFN-α DC vaccine induces allogeneic T cell proliferation. Function of LIPO-5-loaded DC vaccine from HIV-1 patient A3 in allogeneic MLR as measured by CFSE dilution. Controls were T cells with media alone (C-) or with CD3/CD28 dynal beads (C+).

Table 9. Summary of allogeneic MLR by CFSE dilution induced by LIPO-5 loaded monocyte-derived GM-CSF/IFN-α DC vaccines from HIV patients.

DC Vaccine	1:20	1:100	1:500	C-	C+
A1	50.0*	21.0	0.2	0.1	72.0
A2	85.0	31.7	3.8	1.6	87.8
A3	65.0	36.0	8.0	0.3	88.0
A4	25.0	14.0	6.0	0.6	70.4
A5	18.3	10.7	4.9	1.0	55.6
A6	12.6	7.1	3.5	0.4	56.4
Mean	42.8	20.3	4.4	0.9	71.7
Std. Dev.	29.0	12.0	2.6	0.6	14.3

*Values are presented as percentage (%) $CD3^{+}CFSE^{lo}$ T cells.

DC Vaccine Induces HIV-1-Specific $CD4^+$ and $CD8^+$ T Cell Responses

To determine the dose of LIPO-5 required to load the DC, several batches of DC preparations were loaded using a range of LIPO-5 concentrations (from 0.003 μM to 3.0 μM). These differentially loaded DCs were then analyzed for their ability to expand HIV-1 antigen-specific autologous T cells in a ten-day co-culture assay illustrated in Scheme 2A. The expanded cells were then restimulated for 4 hours with the HIV-1-antigen peptides to assess the percentage of responding IFN-γ producing T cells by flow cytometry (Figure 5). The peak responses were observed with T cells that had been co-cultured with DC loaded at LIPO-5 concentrations ≥0.1 μM. Because this finding was consistent across DC batches (Figure 6), the LIPO-5 loading concentration was set at 0.1 μM for manufacture of the HIV-1 therapeutic DC vaccine product targeted for clinical evaluation.

We then evaluated six clinical-grade DC vaccines from HIV-1-infected patients for the ability to elicit HIV-1-antigen-specific T cell responses. One hundred thousand LIPO-5 (0.1 μM)-loaded DC were cultured with $2x10^6$ autologous patient lymphocytes for 10 days in the presence of IL-2 and IL-7. The expanded cells were then restimulated for 4 hours with the five individual HIV-1 peptides (10 μM, no lipidated tail) to assess the percentage of IFN-γ producing T cells by flow cytometry. A fraction of the expanded T cells were cultured without peptides to assess background IFN-γ secretion or with PMA/IONO to assess potential IFN-γ secretion and the validity of the assay.

Comprehensive scatter-graphs and statistical analysis of the antigen-specificity analysis are shown in Figure 7. The analysis began with gating on the viable lymphocyte population (Scheme 3) followed by gating on $CD3^+$ T cells. Further analysis of the gated $CD3^+$ T cells reveals that the specific HIV-1-antigen peptides are recognized by the

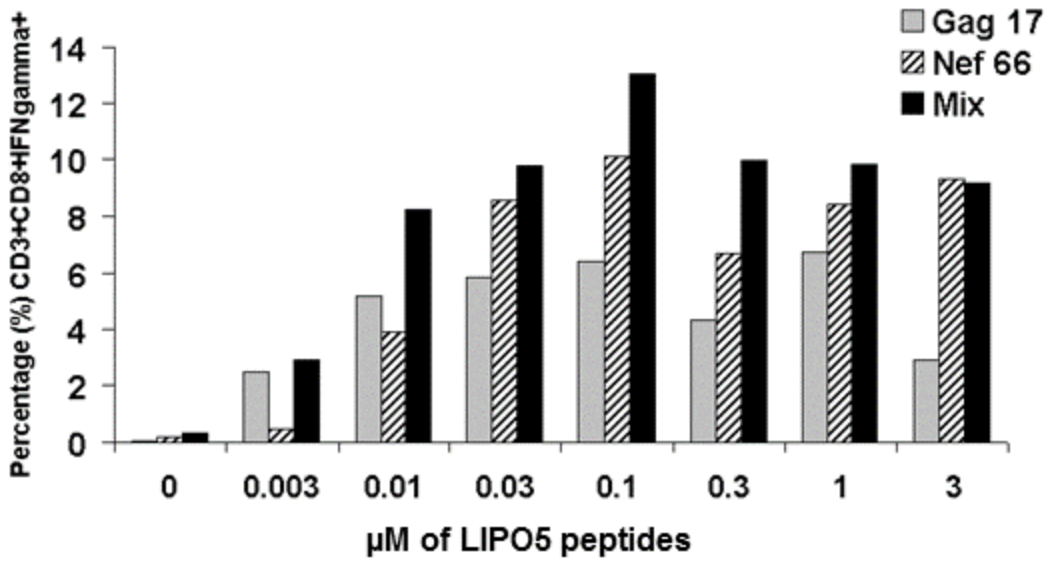


Figure 5. IFN-γ antigen-specific responses peak at 0.1 µM/peptide. DC vaccines loaded with LIPO-5 concentrations ranging from 0.003 µM to 3.0 µM were prepared with monocytes obtained from HIV-1 patient A5. The assay consisted of co-culturing autologous lymphocytes with the different DC vaccines for 10 days, followed by a 4 hour restimulation with the HIV-1-antigen non-lipidated peptides. FACS analysis was used to determine the percentage of IFN-γ-producing $CD8^+$ T cells responding to the $Gag_{17\text{-}35}$ (Gag 17-gray), $Nef_{66\text{-}97}$ (Nef 66-diagonal stripes), and mixture of the five HIV-1-antigen peptides (black). The negative control is the response of T cells that were co-cultured with DC that were not loaded with any HIV-1-antigen peptides.

responding IFN-γ-producing $CD8^+$ or $CD4^+$ T cells. A representative dot plot analysis of single samples is illustrated in Figure 7A. The background IFN-γ-secreting $CD8^+$ and $CD4^+$ T cells ranged from 0.03-0.2% and 0-0.07%, respectively. When DCs are cultured without LIPO-5 and co-cultured with autologous lymphocytes in the same manner, IFN-γ secretion was not detected (Figure 5). IFN-γ secretion was seen when cells were cultured with PMA/IONO (data not shown). Figure 7B reports the comprehensive analysis of the average (duplicate or triplicate) percentage of $CD3^+CD8^+IFN\text{-}\gamma^+$ and $CD3^+CD8^-IFN\text{-}\gamma^+$ for patients A1-A6. Positive peptide responses are identified as those greater than three standard deviations from the mean of negative controls. $CD8^+$ positive peptides had to be greater than 0.28% and $CD4^+$ positive peptides had to be greater than 0.10%. Patient A1

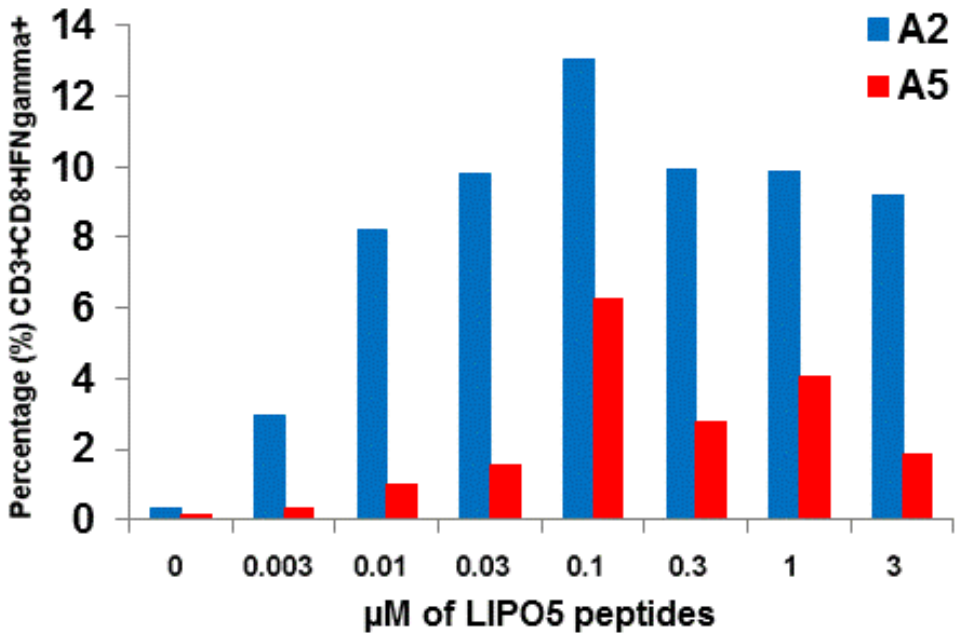


Figure 6. IFN-γ antigen-specific responses peak at 0.1 µM/peptide. DC vaccines loaded with LIPO-5 concentrations ranging from 0.003 µM to 3.0 µM were prepared with monocytes obtained from HIV-1 patient A2 and A5. The assay consisted of co-culturing autologous lymphocytes with the different DC vaccines for 10 days, followed by a 4 hour restimulation with the HIV-1-antigen non-lipidated peptides. FACS analysis was used to determine the percentage of IFN-γ-producing $CD8^+$ T cells responding to the Gag_{17-35} (Gag 17-gray), Nef_{66-97} (Nef 66-diagonal stripes), and mixture of the five HIV-1-antigen peptides (black). The negative control is the response of T cells that were co-cultured with DC that were not loaded with any HIV-1-antigen peptides.

displayed $CD8^+$ T cell responses to Nef 66, Nef 116, and Pol 325. Patient A2 displayed $CD8^+$ T cell responses to Nef 116 and $CD4^+$ T cell responses to Gag 253. Patient A3 displayed $CD8^+$ T cell responses to Nef 116. Patient A4 displayed $CD8^+$ T cell responses to Gag 253, Nef 66, Nef 116 and $CD4^+$ T cell responses to Gag 17 and Gag 253. Patient A5 displayed $CD8^+$ T cell responses to Gag 17, Gag 253, and Nef 66 and $CD4^+$ T cell responses to Gag 17 and Nef 66. Patient A6 displayed $CD8^+$ T cell responses to Gag 253, Nef 66 and $CD4^+$ T cell responses to Gag 253, Nef 66, and Pol 325. Interestingly, Nef 66 and Nef 116 were the most frequent $CD8^+$ T cell responses, as seen in four of the six patients based on IFN-γ secretion. This assay demonstrated that $CD8^+$ T cell

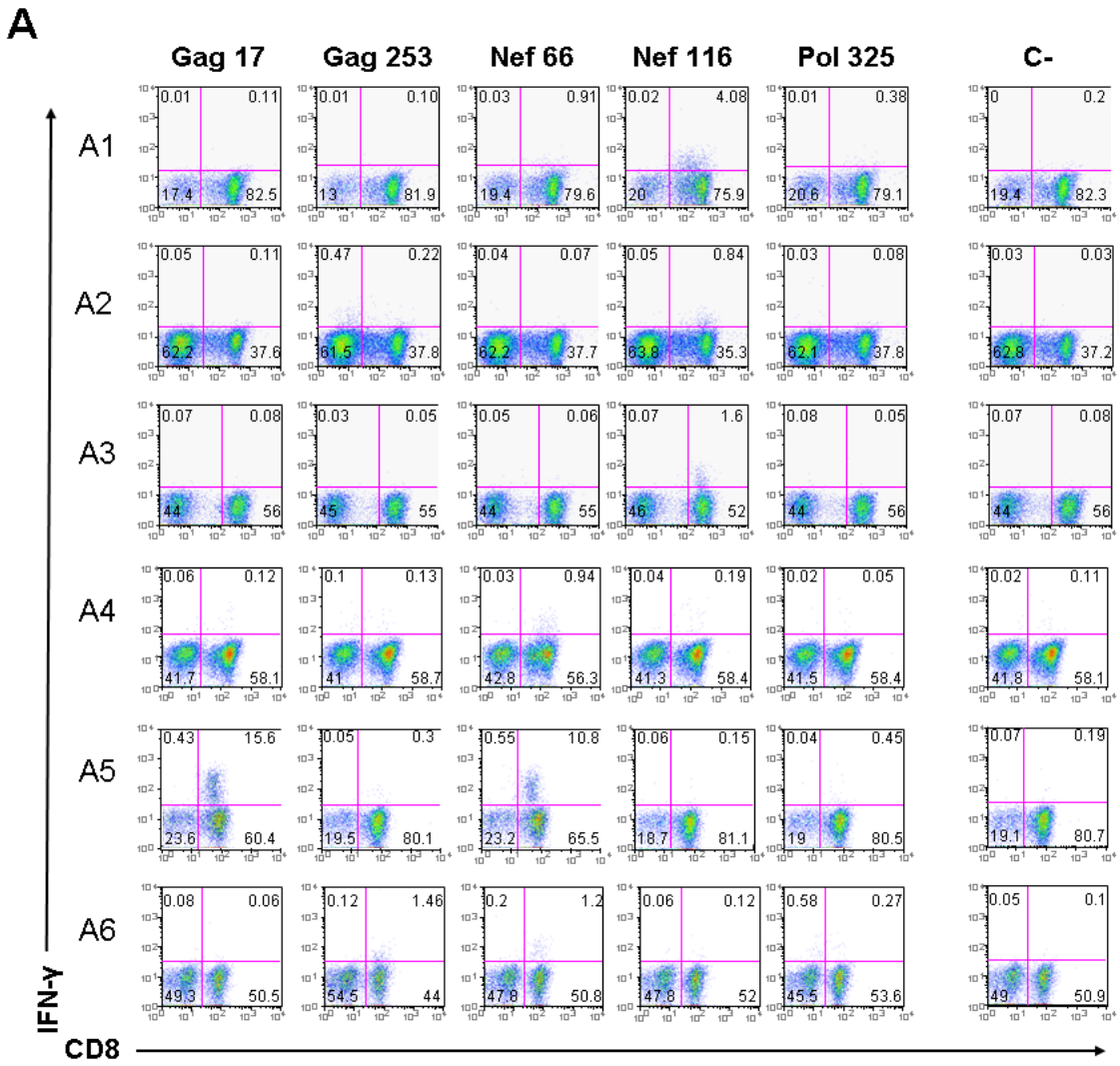


Figure 7. Compilation of IFN-γ antigen-specific T cell elicited by LIPO-5-loaded GM-CSF/IFN-α DC vaccine. ANRS HIV-LIPO-5-loaded DC vaccines induce autologous T cells to respond to specific HIV-1-antigen peptides. FACS analysis was performed to determine the percentage of DC-vaccine-expanded autologous T cells that respond to the specific HIV-1-antigen peptides in LIPO-5 ($Gag_{17\text{-}35}$ [Gag 17], $Gag_{253\text{-}284}$ [Gag 253], $Nef_{66\text{-}97}$ [Nef 66], $Nef_{116\text{-}145}$ [Nef 116] and $Pol_{325\text{-}355}$ [Pol 325]). Panel A, $CD8^+$ and $CD8^-$ T cells that produced IFN-γ were identified as responding to HIV-1-antigen peptides. The analysis was conducted with DC vaccine prepared from six HIV-1 patients (A1-A6).

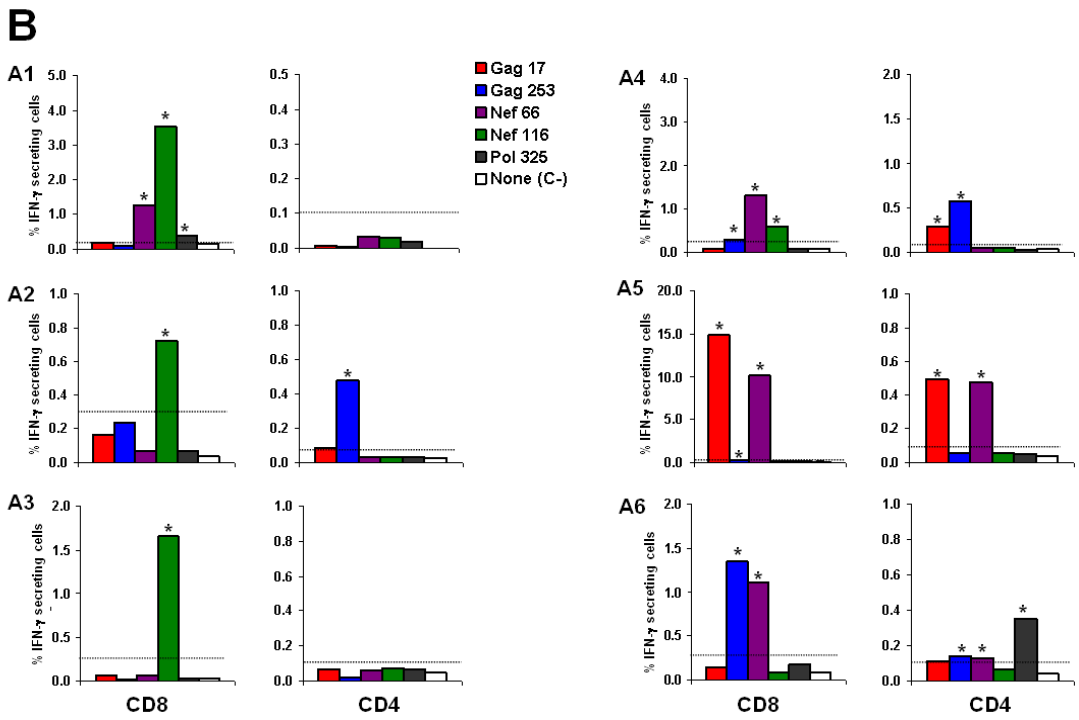


Figure 7. Compilation of IFN-γ antigen-specific T cell elicited by LIPO-5-loaded GM-CSF/IFN-α DC vaccine. ANRS HIV-LIPO-5-loaded DC vaccines induce autologous T cells to respond to specific HIV-1-antigen peptides. FACS analysis was performed to determine the percentage of DC-vaccine-expanded autologous T cells that respond to the specific HIV-1-antigen peptides in LIPO-5 ($Gag_{17\text{-}35}$ [Gag 17], $Gag_{253\text{-}284}$ [Gag 253], $Nef_{66\text{-}97}$ [Nef 66], $Nef_{116\text{-}145}$ [Nef 116] and $Pol_{325\text{-}355}$ [Pol 325]). Panel B reports the average percentage of $CD3^+CD8^+IFN\text{-}\gamma^+$ and $CD3^+CD8^-IFN\text{-}\gamma^+$ for patients A1-A6. Positive peptide responses are identified as those greater than three standard deviations from the mean of the negative controls.

responses to the different HIV-1 antigens in the vaccine could be elicited by all of the HIV-1 antigen lipopeptides, although each patient had unique $CD8^+$ T cell repertoires and HLA haplotypes.

The DC Vaccine only Activates the Peptide-Specific T Cells

To determine the ability of the DC vaccine to select the HIV-1-specific repertoire, we compared, for two patients (A7 and A8), the breadth of the response to the DC vaccine with that to the single peptides from overlapping HIV Gag and Nef peptide

libraries. As illustrated in Scheme 2, ANRS HIV-LIPO-5-loaded DC vaccines were cultured with autologous lymphocytes for 10 days in the presence of IL-2 Scheme 2A, or PBMC were cultured with individual 15 AA peptides in the presence of IL-2 for 7 days of culture Scheme 2B. After culture, the cells were restimulated with the respective peptides for 48 hours and IFN-γ, IL-10 and IL-13 levels were measured in the supernatants.

The results from the single peptide analysis indicate a broad repertoire of IFN-γ-producing T cells in patients A7 and A8 (Figure 8A). Similar results were seen with IL-13 (Figure 8B). Unlike the single peptides, the DC vaccine decreased the number of expanded IL-10-secreting T cells (Figure 8C). Collectively, these data further demonstrate the efficacy of LIPO-5-loaded DC vaccines to expand in vitro the memory $CD4^+$ and $CD8^+$ T cells in HIV-1 patients undergoing HAART.

Discussion

In this chapter, we demonstrated the ability to scale up manufacturing of the DC vaccines using elutriated monocytes as precursors to generated GM-CSF/IFN-α DC vaccines from HIV patients. This was the pre-clinical development of a process for cGMP manufacture of a frozen autologous DC vaccine derived from monocytes isolated from HAART-treated, HIV-1 infected patients. The process consisted of culturing monocytes with GM-CSF and IFN-α for three days. The resulting DC were then loaded with ANRS HIV-LIPO-5 vaccine and activated with LPS. These DC were shown to express high levels of MHC Class II and co-stimulatory molecules. When tested in an in vitro immune potency assay, the DC vaccines were capable of eliciting allogeneic T cell proliferation and autologous HIV-1 antigen-specific $CD8^+$ and $CD4^+$ T cell responses, as

measured by an increase in the number of IFN-γ-producing T cells but not IL-10. Furthermore, the autologous T cells that were expanded by the DC vaccines were focused on the HIV-1 antigen epitopes expressed with the LIPO-5 lipopeptides.

Previous studies evaluated the use of monocyte-derived DC in SCID mice and human studies verified the ability of these cells to expand HIV-1-specific $CD8^+$ T cells secreting IFN-γ (Carbonneil and others 2003; Connolly and others 2008; Routy and others 2010) and perforin as well as $CD4^+$ T cells secreting IFN-γ and IL-2 (Lu and others 2004). Evaluation of vaccinating hu-PBL-SCID mice (SCID mice reconstituted with healthy donor PBMCs) with GM-CSF/IFN-α DCs has been shown to induce higher numbers of HIV-1-specific $CD8^+$ T cells than those elicited by GM-CSF/IL-4 DCs. Thus, demonstrating that the type of DC used for HIV-1-specific vaccination is critical for the quality and quantity of T cell responses. This study is also evidence of the ability of GM-CSF/IFN-α DC to prime a T cell response in vivo. While this is an important attribute, the intent of the DC vaccine described herein will be used as a therapeutic approach for expanding the T cell response in HIV-1-infected patients in conjunction with HAART. Vaccination in this population will presumably trigger effector and memory T cell responses. As seen in Figure 8A, IFN-γ production by HIV-1-antigen-responsive T cells was seen initially when peripheral blood lymphocytes were challenged with 15 AA HIV-1-antigen peptides and was further enhanced and focused with vaccination of the DC vaccine. Furthermore, the DC vaccine dramatically decreased the induction of IL-10-secreting cells, which have been indicative of T regulatory cells (Figure 8C) (Vignali and others 2008). Exploiting the toll-like receptor 2 signaling property of the palmitoyl-lysylamide lipid tail, it has been previously reported that lipopeptides used in vaccine studies are highly efficient at inducing immune responses,

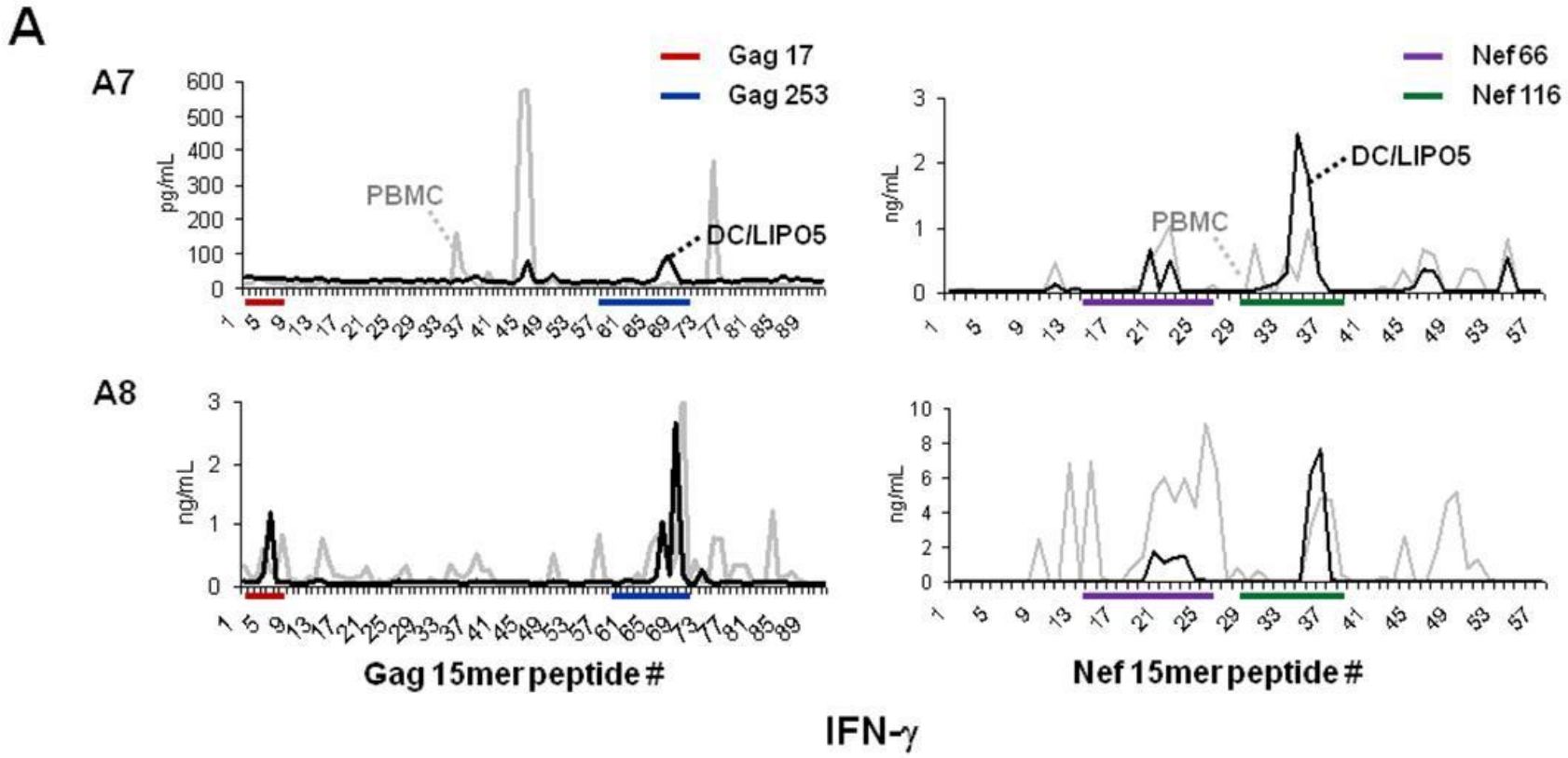


Figure 8. Epitope mapping of antigen-specific T cells. LIPO-5-loaded DC vaccines from patients A7 and A8 focus the autologous T cell response on epitopes within regions of the LIPO-5-antigen peptides. Solid bars along the peptides denote the position where the sequences from the LIPO-5 are located. Red represents Gag 17; Blue represents Gag 253; Purple represents Nef 66; Green represents Nef 116. Autologous T cell responses were assessed by the amount of IFN-γ (A) secreted by cells restimulated with the indicated HIV Gag and Nef peptide libraries. PBMC (light tracings) were primed with the HIV-1-antigen peptide libraries and DC/LIPO-5 (dark tracings) were autologous lymphocytes primed with the DC vaccine.

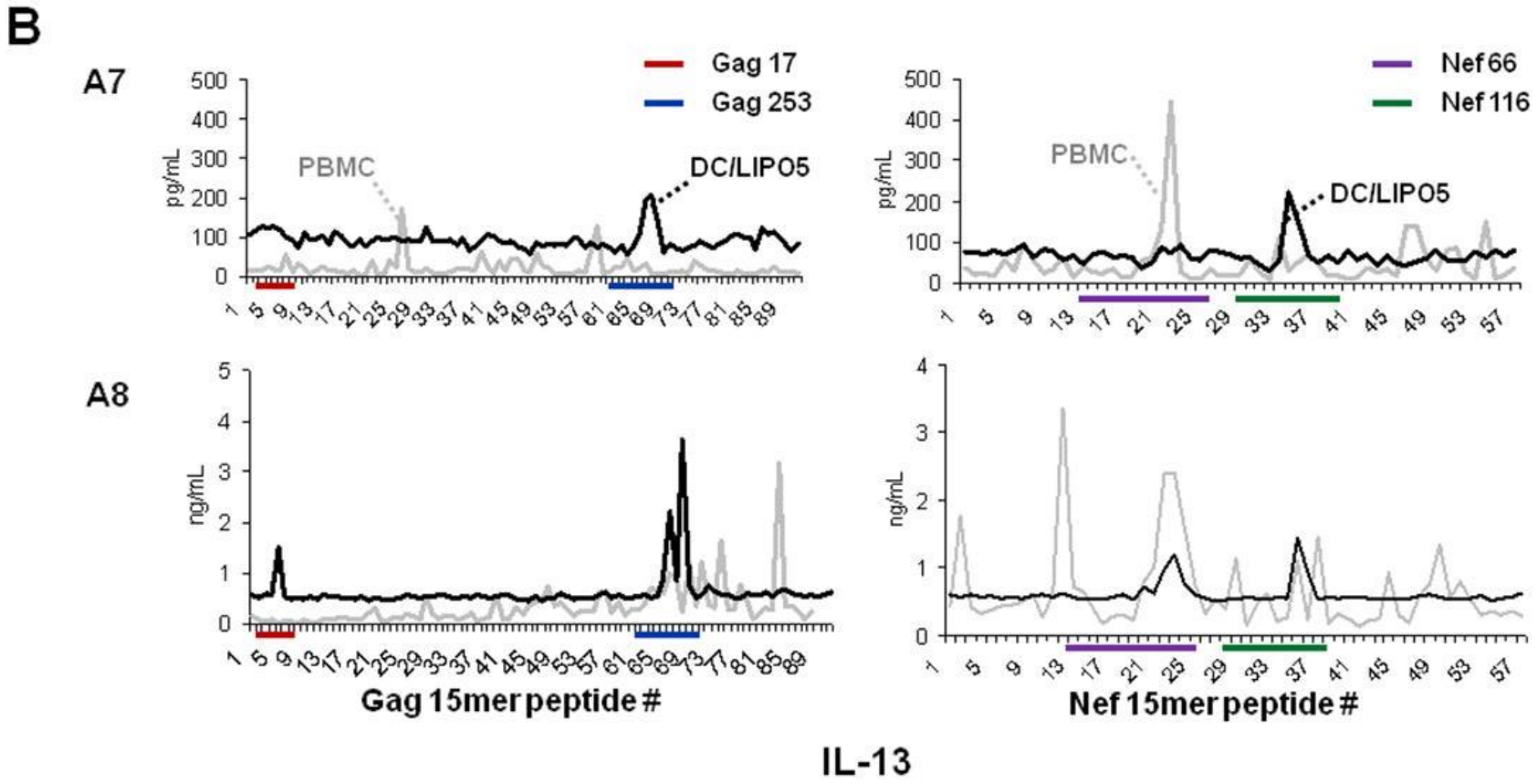


Figure 8. Epitope mapping of antigen-specific T cells. LIPO-5-loaded DC vaccines from patients A7 and A8 focus the autologous T cell response on epitopes within regions of the LIPO-5-antigen peptides. Solid bars along the peptides denote the position where the sequences from the LIPO-5 are located. Red represents Gag 17; Blue represents Gag 253; Purple represents Nef 66; Green represents Nef 116. Autologous T cell responses were assessed by the amount of IL-13 (B) secreted by cells restimulated with the indicated HIV Gag and Nef peptide libraries. PBMC (light tracings) were primed with the HIV-1-antigen peptide libraries and DC/LIPO-5 (dark tracings) were autologous lymphocytes primed with the DC vaccine.

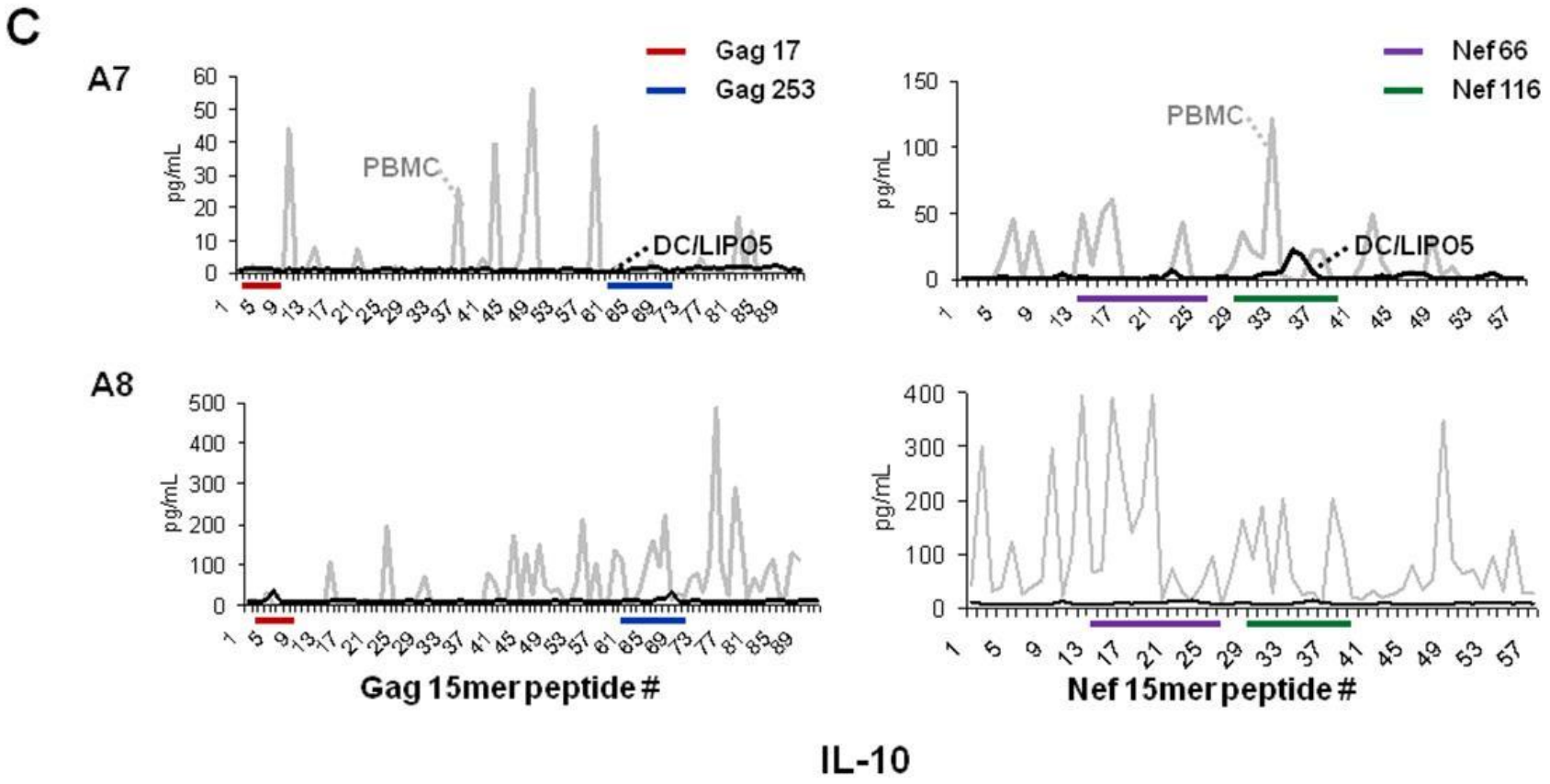


Figure 8. Epitope mapping of antigen-specific T cells. LIPO-5-loaded DC vaccines from patients A7 and A8 focus the autologous T cell response on epitopes within regions of the LIPO-5-antigen peptides. Solid bars along the peptides denote the position where the sequences from the LIPO-5 are located. Red represents Gag 17; Blue represents Gag 253; Purple represents Nef 66; Green represents Nef 116. Autologous T cell responses were assessed by the amount of IL-10 (C) secreted by cells restimulated with the indicated HIV Gag and Nef peptide libraries. PBMC (light tracings) were primed with the HIV-1-antigen peptide libraries and DC/LIPO-5 (dark tracings) were autologous lymphocytes primed with the DC vaccine.

including anti-HIV-1 responses (Hosmalin and others 2001; Martinon and others 1992; Zhu and others 2004). The ANRS HIV-LIPO-5 used in the development of the DC vaccine reported in this study has been evaluated in numerous clinical trials, resulting in multiepitopic HIV-1-antigen-specific T cell responses (Goujard and others 2007; Launay and others 2007; Pialoux and others 2001; Pialoux and others 2008; Salmon-Ceron and others 2010). Based on the Los Alamos database and ANRS data, each of the HIV-1 antigen peptides present in LIPO-5 are recognized by numerous HLA haplotypes (Table 10 and 11) and have been documented to be part of conserved regions of HIV-1 antigens (Salmon-Ceron and others 2010). This is an important criterion for the development of a vaccine that can treat as many patients as possible with little immunological restrictions due to HLA haplotype differences. Furthermore, it should be noted that the frequency of the T cell precursors are variable per patient which will result in different epitopes presented (as seen in Figure 7B).

Combining the two strategies, GM-CSF/IFN-α DC and LIPO-5, has become an attractive union for inducing a broad potent effector immune response (Cobb and others 2011), which is the first to be tested in humans. Thus, a Phase I/II clinical trial has been initiated and is nearing completion (clinicaltrials.gov identifier: NCT00796770) for therapeutic vaccination in chronically HIV-1-infected patients on HAART. In the next chapter we explore activation of the GM-CSF/IFN-α DC with anti-CD40 antibodies for inducing a stronger $CD8^+$ T cell response.

Table 10. HLA Class I A-, B-, C- haplotypes with predicted ability to recognize and present epitopes of LIPO-5 peptides.

	Class I-CTL Epitopes		
Peptides	A	B	C
Gag $_{17-35}$	2,11,23,24,30,31,68	7,8,27,42,62	w4
Gag $_{253-284}$	2,3,11,24,33	7,8,15,27,35,51,52,53,62	w18
Nef $_{66-97}$	2,3,11,24,30,68	7,8,14,27,35,42,51,53,57,58,62,63,81	w4,w8
Nef $_{116-145}$	1,2,3,23,24,29,30,32,33,68	7,15,17,18,27,35,37,42,49,51,53,54,57,58,62,63,81	w6
Pol $_{325-355}$	2,3,11,24,30,33,68	7,15,18,35,44,51,53	n/a

Table 11. HLA Class II DR haplotypes with predicted ability to recognize and present epitopes of LIPO-5 peptides.

Peptides	Class II-DR : Helper Epitopes
Gag $_{17-35}$	13
Gag $_{253-284}$	B1*1,4,7,11,13,15,B5*1,4
Nef $_{66-97}$	w15,1
Nef $_{116-145}$	n/a
Pol $_{325-355}$	n/a

CHAPTER FOUR

Dendritic Cells and Lipopeptides induced Immunity against AIDS – DALIA 1

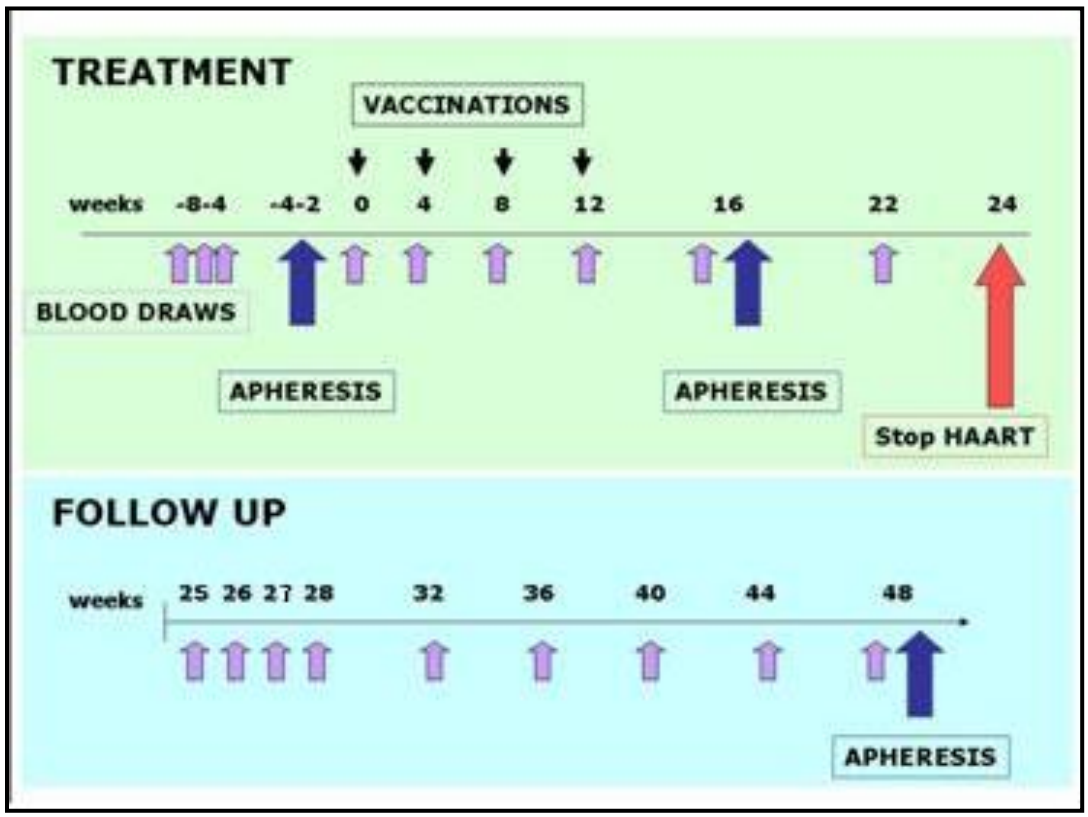
Rationale

In Chapter 3 we demonstrated that monocytes from HIV patients functioned as viable precursors to generate GM-CSF/IFN-α DC vaccines. The DC vaccines were equipped with classical DC phenotypic characteristics and co-stimulatory molecules. Giemsa staining revealed the DCs to have dendrites that allow enhanced cellular cross-talk. Multiplex bead-based assays detected a unique cytokine/chemokine profile of the DC vaccines among which IL-12p40 was detected and is important for the development for cytotoxic T cells. When GM-CSF/IFN-α DC vaccines were pulsed with ANRS LIPO-5 peptides, they elicited potent allogeneic lymphocyte proliferation and antigen-specific $CD8^+$ and $CD4^+$ T cells as measured by IFN-γ detection assays. This chapter outlines the Phase I/II clinical trial in HIV infected patients on HAART that uses the vaccine subject to development and preclinical validation outlined in the previous chapters.

The primary objective is to evaluate the effect on HIV-specific immunity of adding ex vivo generated interferon-α DCs loaded with HIV-1 lipopeptides and activated with lipopolysaccharide (BIIR/ANRS-HIVax-001, the DC vaccine product) over a period of 16 weeks in HIV-1 infected patients who have been receiving antiretroviral therapy for at least 12 months with HIV-1 RNA <50 copies/mL and $CD4^+$ T cell counts >500/mm^3.

Clinical Trial Outline

This clinical trial is a phase I/II, single-center study in HIV infected patients with 19 patients. Patients are consented and undergo three cell collections by apheresis; before vaccination, after vaccination, and after antiretroviral treatment interruption (ATI). Study visits take place at weeks -8/-4 (screening), -4/-2 (apheresis/vaccine manufacture), 0 (vaccination 1), 4 (vaccination 2), 8 (vaccination 3), 12 (vaccination 4), 16, 22 (immunological evaluation), 24 (ATI, if HIV-1 RNA < 400 copies/mL), 28, 32, 36, 40, 44 (monitoring) and 48 (final evaluation) (Scheme 5).



Scheme 5. DALIA 1 clinical trial vaccination, blood draw, and apheresis schedule.

Preliminary Results

Purity of Monocytes Enriched in Fraction 5 from HIV Patients

The monocyte purity of the elutriated Fraction 5 which is used as the starting cell population for the DC vaccines is reported in Table 18. The values are determined by ABX Pentra 60C+ (Horiba ABX Diagnostics, Montipellier, France) analysis of the elutriated cells in monocyte enriched F5. Excluding D1-3, D1-4, D1-6, D1-10, and D1-12, the patients had monocyte purity of ≥83% (range: 83.2-96.7) with 0.6-5.8% neutrophil and 1.1-11.2% lymphocyte contamination. D1-3, D1-4, D1-6, D1-10, and D1-12 had monocyte purity that ranged 52.2-79.3% with 2.8-31% neutrophil and 3.0-42.2% lymphocyte contamination. Other contaminating cell types in F5 for these patients consisted of lymphocytes ranging from 1.7% to 11.2%, eosinophils ranging from 0% to 1.4%, and basophils ranging from 1.1% to 2.2%.

Generation of GM-CSF/IFN-α DC Vaccines

The FACS plots in Figure 9 below provide an example of the typical DC vaccine phenotype. These plots are of DC vaccines D1-17. The cell surface phenotype of the DC vaccines is summarized below in Table 13. Antigen-loaded DC vaccines were manufactured in three-day monocyte culture with GM-CSF and IFN-α, as described in Material and Methods and illustrated in Scheme 4. Thawed DC vaccines were used to conduct the characterization assays described below. The DC vaccines had a high frequency of HLA-DR^+/$CD11c^+$ (mean, 95.5%; range, 83-99%), $CD14^+$ (mean, 90.3%; range, 74-95%) and $CD80^+$ cells (mean, 91.9%; range, 83-97%). Most DCs expressed $CD1b/c^+$ (mean, 56.9%; range, 25-76%) and $CD83^+$ (mean, 73.0%; range, 39-95%).

Thus, the DC vaccines express surface molecules characteristic of DC with co-stimulatory molecules necessary for T cell interaction.

Table 12. Distribution of cells in elutriated fraction 5 collected from HIV-1 infected patients in DALIA 1 clinical trial as measured by ABX.

DC Vaccine	Monocytes	Lymphocytes	Neutrophils	Eosinophils	Basophils
D1-1	90.4*	7.6	0.8	0.0	1.2
D1-2	92.8	5.4	0.6	0.0	1.2
D1-3	73.2	5.2	18.8	0.6	2.2
D1-4	62.2	8.8	27.1	0.6	1.3
D1-5	95.9	1.7	0.8	0.0	1.6
D1-6	63.0	3.0	30.9	1.4	1.7
D1-7	85.4	11.2	2.2	0.1	1.1
D1-8	90.7	3.9	3.7	0.0	1.7
D1-9	89.8	7.5	1.8	0.0	0.9
D1-10	79.3	16.0	2.8	0.0	1.9
D1-11	89.4	7.5	2.1	0.1	0.9
D1-12	52.2	42.2	5.3	0.0	0.3
D1-13	88.4	7.0	3.2	0.1	1.3
D1-14	83.2	9.8	5.0	0.0	2.0
D1-15	87.5	9.7	1.1	0.0	1.7
D1-16	96.7	1.1	1.0	0.0	1.2
D1-17	94.5	1.6	2.8	0.1	1.0
D1-18	90.5	2.2	5.8	0.0	1.5
D1-19	88.4	5.4	5.0	0.1	1.1
Mean	83.9	8.3	6.4	0.2	1.4
Std. Dev.	12.47	9.06	8.95	0.35	0.45

*Values represent the percentage of cells type.

DALIA 1 GM-CSF/IFN-α DC Vaccines Elicit Allogeneic Lymphocyte Proliferation

Allogeneic T cells collected from normal healthy donors were stained with CFSE. One hundred thousand lymphocytes were cultured with the DC vaccine added at various ratios from 1:20 to 1:303. After 5 days, the cells were stained with fluorescence-conjugated anti-CD3 (PerCP) and anti-CD8 (APC) and analyzed by flow cytometry.

Figure 9. Representative DC phenotype by dot plot analysis of LIPO-5 loaded GM-CSF/IFN-α DC vaccine. FACS analysis dot-plots of the phenotype of ANRS HIV-LIPO-5-loaded DC vaccine from HIV-1 patient D1-7.

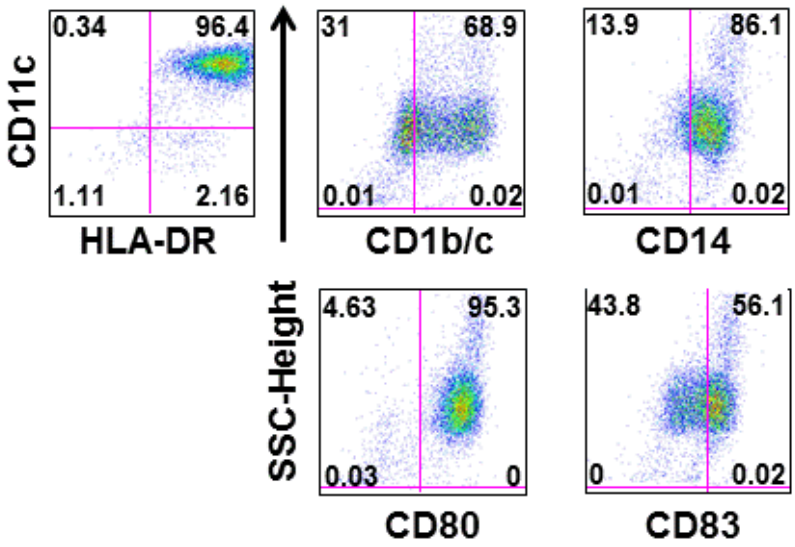


Table 13. Summary of phenotypes of DALIA 1 GM-CSF/IFN-α DC vaccines prepared with monocytes from HIV-1 infected patients D1-1-D1-19.

DC Vaccine	HLA-DR$^+$/CD11c$^+$	CD1b/c$^+$	CD14$^+$	CD80$^+$	CD83$^+$
D1-1	95.7*	51.6	91.3	94.2	68.3
D1-2	95.3	47.5	94.2	95.2	72.4
D1-3	90.4	52.3	87.1	92.7	49.7
D1-4	94.2	59.9	84.9	92.6	70.7
D1-5	97.9	54.5	93.7	96.7	95.2
D1-6	82.5	36.2	82.7	82.7	79.5
D1-7	97.1	63.5	92.7	92.2	91.6
D1-8	97.2	64.7	93.0	94.4	93.9
D1-9	98.0	65.4	92.0	95.4	84.3
D1-10	97.6	57.9	94.7	96.3	92.2
D1-11	96.5	75.8	92.5	96.4	90.0
D1-12	93.7	52.5	73.5	84.5	39.0
D1-13	98.7	40.2	95.2	88.7	64.6
D1-14	97.2	49.2	93.8	96.3	92.5
D1-15	97.6	25.1	90.3	80.5	76.6
D1-16	98.5	72.3	87.1	85.4	66.9
D1-17	96.4	68.9	86.1	95.3	56.1
D1-18	96.3	69.4	93.9	95.6	60.7
D1-19	94.1	73.5	81.1	91.0	43.2
Mean	95.5	56.9	90.3	91.9	73.0
Std. Dev.	3.75	13.54	4.49	5.09	17.73

*Values represent the percentage of cells in the DC vaccine that express the specified cell surface marker(s).

Figure 10 represents a dot plot analysis of the MLR assay performed with the DC vaccine from HIV-1 patient D1-17. Figure 11 and Table 14 reports the allogeneic T cell responses of the $CD3^+$ population for HIV-1 patient DC vaccines (D1-1-D1-19).

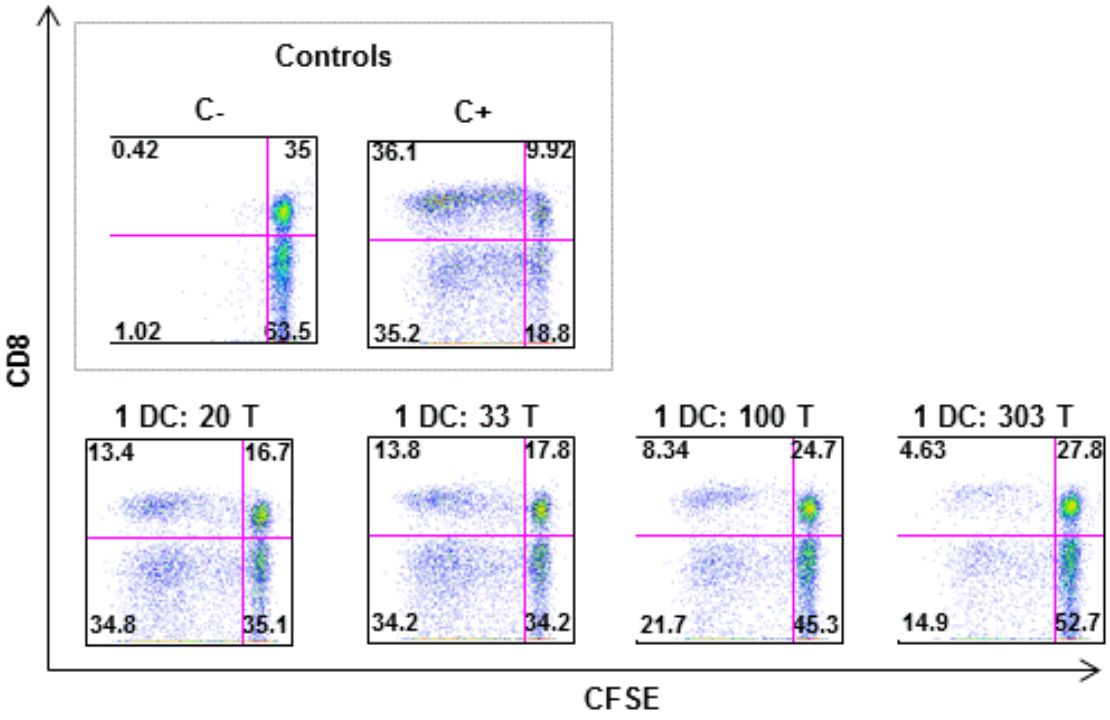


Figure 10. Dot plot representation of LIPO-5 loaded GM-CSF/IFN-α DC vaccine-induced allogeneic T cell proliferation. Function of LIPO-5-loaded DC vaccine from HIV-1 patient D1-17 in allogeneic MLR as measured by CFSE dilution.

At a ratio of 1:20, a mean of 23.9% of $CD3^+$ T cells dilute CFSE (range: 3.4-49.7%). At a ratio of 1:33, 20.9% of $CD3^+$ T cells diluted CFSE (range: 3.9-44.8%). At a ratio of 1:100, 13.6% of $CD3^+$ T cells diluted CFSE (range: 3.9-33.3%). CD3/CD28 dynal beads used as a positive control, a mean of 69.8% the proliferated $CD3^+$ T cells diluted CFSE. The proliferation background (C-) mean was 1.51%. Thus, monocyte-derived GM-CSF/IFN-α, ANRS HIV-LIPO-5 loaded, LPS-activated DC vaccines

generated from HIV-1 patients act as potent antigen presenting cells in the allogeneic MLR.

Table 14. Summary of allogeneic MLR $CD3^+$ T cell proliferation by CFSE dilution induced by LIPO-5 loaded monocyte-derived GM-CSF/IFN-α DC vaccines from HIV patients.

DC Vaccine	1:20	1:33	1:100	1:303	C+	C-
D1-1	21.9*	17.5	12.1	5.5	62.17	1.07
D1-2	27.7	24.4	16.3	5.8	63.93	0.86
D1-3	21.7	18.7	12.3	7.2	63.2	0.86
D1-4	27.4	23.2	14.1	6.0	63.4	2.35
D1-5	26.3	21.0	13.2	7.3	58.2	0.66
D1-6	10.8	9.4	5.6	3.5	48.93	0.87
D1-7	9.8	10.2	8.9	8.5	73.17	8.09
D1-8	11.1	7.2	4.9	2.0	79.73	0.33
D1-9	14.8	14.5	6.4	2.3	84.83	0.25
D1-10	3.4	3.9	3.5	1.8	74.5	1.17
D1-11	16.9	14.2	8.4	4.9	69.17	0.26
D1-12	42.7	39.8	23.7	8.7	79.13	0.25
D1-13	19.7	16.2	10.3	6.6	73.1	0.45
D1-14	7.8	6.4	3.9	1.7	74.7	0.68
D1-15	49.7	43.6	33.3	20.5	64.9	0.7
D1-16	36.7	29.4	17.4	13.0	75.33	8.14
D1-17	47.7	44.8	31.0	20.5	72.5	1
D1-18	25.5	22.0	16.4	5.9	72.73	0.15
D1-19	33.2	31.3	17.17	5.8	71.8	0.54
Mean	23.9	20.9	13.6	7.2	69.8	1.5
Std. Dev.	13.41	12.25	8.47	5.43	8.51	2.38

*Values are presented as percentage (%) $CFSE^{lo}CD3^+CD8^+$T cells.

GM-CSF/IFN-α DC Vaccines Elicit Immune Responses

Patient PBMC samples are retained at various time-points throughout the duration of the clinical trial. Immuno-monitoring of the samples retrieved before and after vaccination have revealed that immune responses are elicited. Figure 13 summarized ICS analysis of 16 patient responses either before (A1) or after vaccination (A2). After a

48 hour challenge with a mix of non-LIPO-5, $CD4^+$ (top row) and $CD8^+$ T cells (bottom row) were analyzed for the expression of IFN-γ, TNF-α and IL-2. There is a general trend in the increase of LIPO-5-specific $CD4^+$ T cells after vaccination that is observed for all three cytokines. Conversely, $CD8^+$ T cells show a similar, yet less pronounced increase IFN-γ, TNF-α and IL-2 after vaccination. Furthermore, as a goal of eliciting polyfunctional T cell responses in patients to mimic LTNP, Figure 13 illustrates HIV-specific-$CD4^+$ T cell expressing both IFN-γ and TNF-α after vaccination (A1).

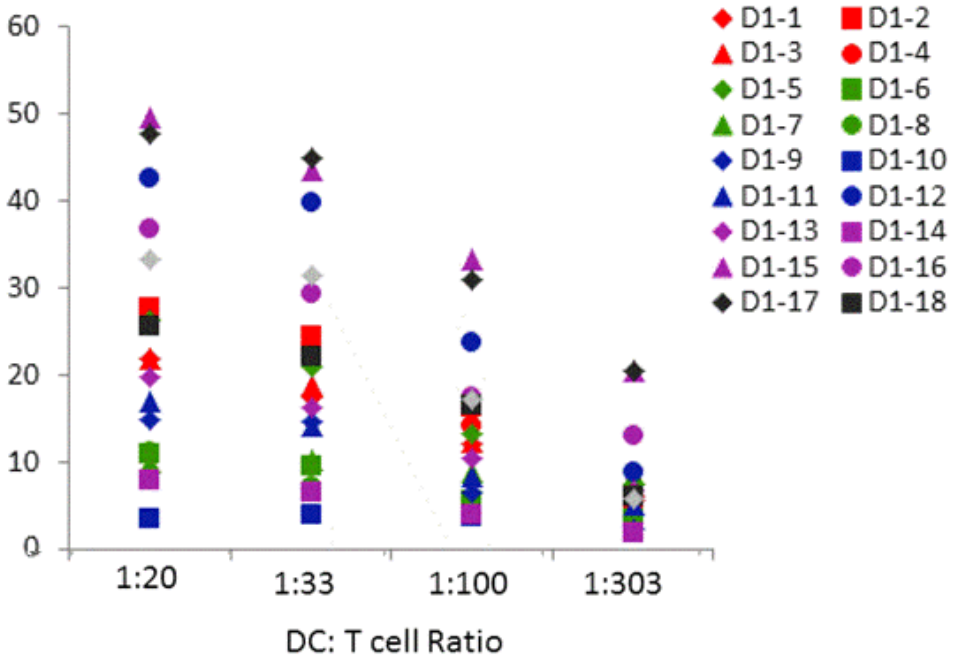


Figure 11. Comparison of LIPO-5 loaded GM-CSF/IFN-α DC vaccine-induced allogeneic $CD3^+$ T cell proliferation.

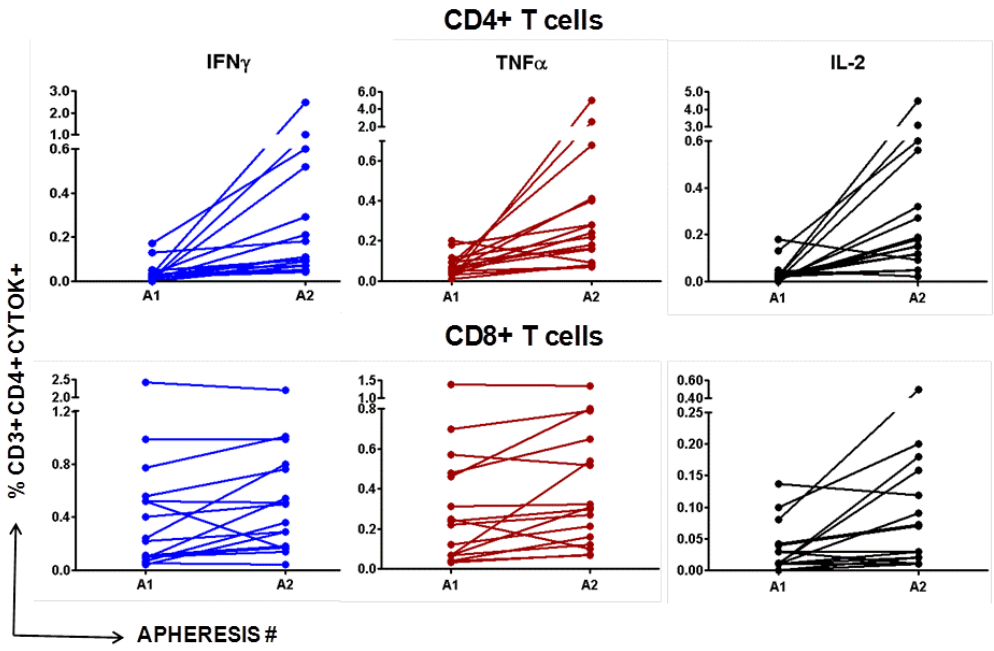


Figure 12. Summary of ICS analysis of sixteen DALIA 1 vaccinated patients in response to non-LIPO-5 peptide mix-stimulated. Top row $CD4^+$ T cells and bottom row $CD8^+$ T cells from HIV patient PBMCs before (A1) and after (A2) vaccination.

Discussion

This chapter demonstrates the monocyte-derived GM-CSF/IFN-α DC vaccines generated by HIV infected patients on HAART resemble the preclinical GM-CSF/IFN-α DCs reported in Chapter 3. The DC vaccines also expressed high levels of MHC Class II and co-stimulatory molecules. When tested in an in vitro immune potency assay, the DC vaccines were capable of eliciting allogeneic T cell proliferation. Furthermore, after vaccination of patients, an increase of HIV-specific responses was observed as measured by the expression of IFN-γ, TNF-α and IL-2. Analyses of these patients are ongoing.

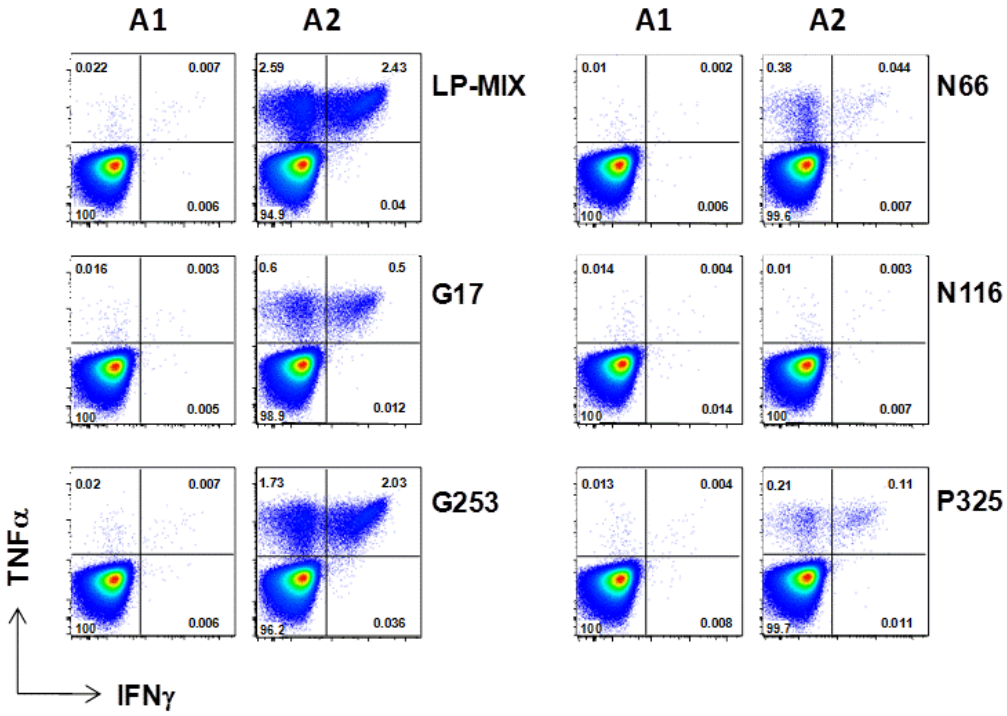


Figure 13. Patient D1-11 analysis of intracellular IFN-γ and TNF-α in long peptide-stimulated $CD4^+$ T cells from HIV patient PBMCs before (A1) and after (A2) vaccination.

CHAPTER FIVE

Preclinical Development of Therapeutic DC for HIV-Infected Patients – GM-CSF/IL-15 DC

Rationale

Studies have shown that GM-CSF and IL-15 DCs elicit potent $CD8^+$ T cells (Dubsky and others 2007). However, this has not been demonstrated in HIV patients. This chapter focuses on the preclinical development of monocyte-derived GM-CSF/IL-15 DC vaccines using HIV patient frozen monocytes and characterizing the function and potency to elicit allogeneic T cell proliferation and autologous antigen-specific T cell responses.

Results

Purity of Monocytes Enriched in Fraction 5 from HIV Patients

The purity of the fraction 5 which is used as the starting product for the DC vaccines is reported in Table 15. The values are determined by ABX Pentra 60C+ (Horiba ABX Diagnostics, Montipellier, France) analysis of the elutriated cells in monocyte enriched F5. Patients D1-1, D1-2, D1-5, D1-7, and D1-8 had monocyte purity of ≥85% with 0.6-3.7% neutrophil contamination. Patients D1-3, D1-4, and D1-6 had 62-73% monocyte purity with 18-31% neutrophil contamination. Other contaminating cell types in F5 for these patients consisted of lymphocytes ranging from 1.7% to 11.2%, eosinophils ranging from 0% to 1.4%, and basophils ranging from 1.1% to 2.2%.

Table 15. Distribution of cells in elutriated fraction 5 collected from HIV-1 infected patients as measured by ABX.

DC Vaccine	Monocytes	Lymphocytes	Neutrophils	Eosinophils	Basophils
D1-1	90.4*	7.6	0.8	0.0	1.2
D1-2	92.8	5.4	0.6	0.0	1.2
D1-3	73.2	5.2	18.8	0.6	2.2
D1-4	62.2	8.8	27.1	0.6	1.3
D1-5	95.9	1.7	0.8	0.0	1.6
D1-6	63.0	3.0	30.9	1.4	1.7
D1-7	85.4	11.2	2.2	0.1	1.1
D1-8	90.7	3.9	3.7	0.0	1.7
Mean	81.7	5.9	10.6	0.3	1.5
Std. Dev.	13.61	3.17	12.88	0.50	0.37

*Values presented as percentage of cell type.

Table 16. Summary of GM-CSF/IL-15 DC vaccine recovery and viability after culture for HIV-1 infected patients D1-1-D1-8.

DC Vaccine	Recovered DCs ($x10^6$)	Percent Recovery (%)	Percent Viability (%)
D1-1	8.4	16.8	82.0
D1-2	6.6	13.2	85.0
D1-3	13.8	27.6	96.0
D1-4	12.0	24.0	83.0
D1-5	16.8	33.6	90.0
D1-6	3.0	6.8	83.0
D1-7	27.6	55.2	92.0
D1-8	81.8	78.4	21.0
Mean	12.5	25.2	86.8
Std. Dev.	7.47	14.80	5.23

Generation of IL-15 DC from HIV Monocytes

The recovery and viability of the cells in the DC vaccines are reported below in Table 16. All of the DC vaccine cultures started with $50x10^6$ monocytes. At the end of the DC vaccine 4 day process the harvested cell concentrations ranged from $3\text{-}28x10^6$

viable cells. While the percentage of viable cells were similar across the 8 DC vaccines (range: 82-96%), the overall number of cells recovered were low for some DC vaccines (range: 7-55%).

The FACS plots in Figure 14 below provide an example of the typical DC vaccine phenotype. These plots are of DC vaccines D1-7. The cell surface phenotype of the DC vaccines is summarized below in Table 17. CD1a staining was not done for DC vaccines D1-3 and D1-5.

DC vaccines generated from patients D1-1, D1-2, D1-5, D1-7 and D1-8, which had a good monocyte purity in elutriation Fraction 5, had similar phenotypes for HLA-DR^+/$CD11c^+$ (98.3-99.9%), $CD1a^+$ (71.1-91.6), $CD1b/c^+$ (79.8-99.2%), $CD207^+$ (16.8-34.3%), $CD80^+$ (88.3-99.6%), and $CD86^+$ (89.3-98.1%). However, the vaccines differed in their expression of $CD14^+$ (1.0-24.8%) and $CD83^+$ (2.6-64.4%).

DC vaccine generated from patients D1-3, D1-4 and D1-6 had high neutrophil contamination of elutriation Fraction 5 prior to freezing. The three DC have similar phenotypes for HLA-DR^+/$CD11c^+$ (85-8-97.0%), $CD83^+$ (7-13%), and $CD86^+$ (92.1-98.7%). However, they have different expressions of CD1a (29.1-46.4%), CD1b/c (60.9-94.1%), CD207 (3.2- 20.5%), $CD14^+$ (4.8-67.5%), and CD80 (0.3-6.8%).

In the current HIV DC vaccine clinical trial, BIIR/ANRS-HIVax-001 which is testing the GM-CSF/IFN-α DC vaccine developed in Chapter 3, the DC phenotype release criteria requires the vaccine to have ≥80% of double positive cells for HLA-DR^+/$CD11c^+$ and ≥70% of CD80. According to the results of the DC phenotype, all of the vaccines meet the HLA-DR^+/$CD11c^+$ QC release requirements. All vaccines except

D1-3, D1-4, and D1-6 also meet the CD80 QC release criteria. Based on the phenotype criteria these three vaccines would not be released for human use.

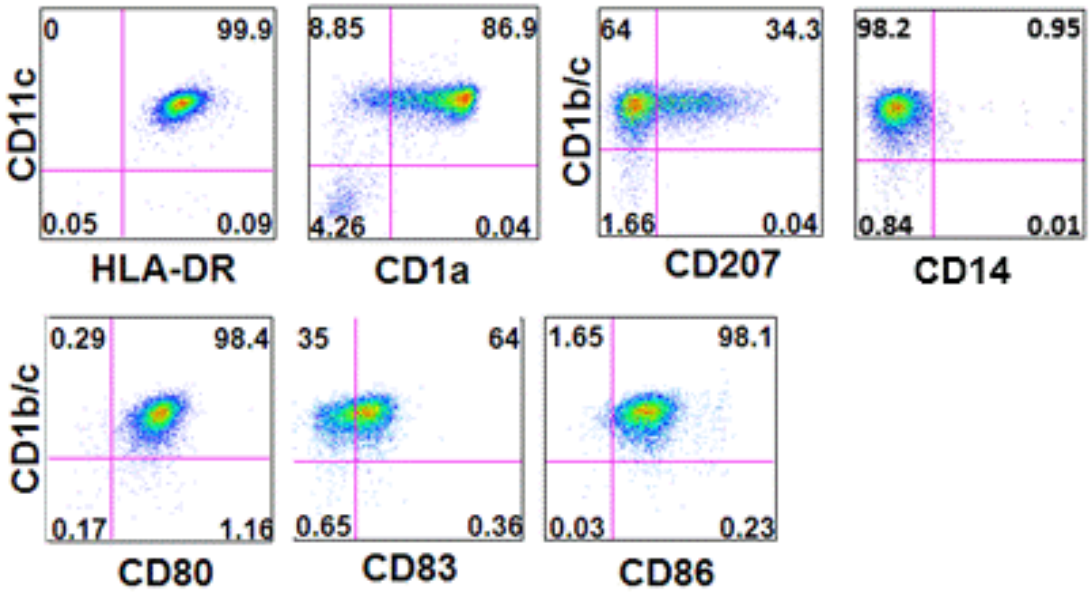


Figure 14. Representative DC phenotype by dot plot analysis of LIPO-5 loaded GM-CSF/Il-15 DC vaccine. FACS analysis dot-plots of the phenotype of ANRS HIV-LIPO-5-loaded DC vaccine from HIV-1 patient D1-7.

Table 17. Summary of phenotypes of GM-CSF/Il-15 DC vaccines prepared with monocytes from HIV-1 infected patients D1-1-D1-8.

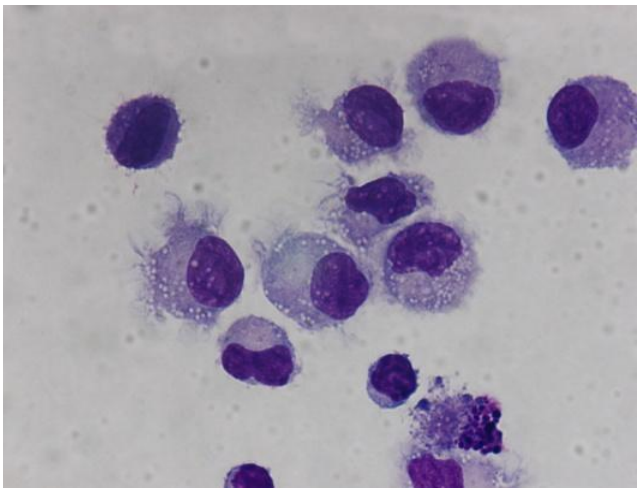
DC Vaccine	HLA-DR$^+$/ CD11c$^+$	CD1a$^+$	CD1b/c$^+$	CD207$^+$	CD80$^+$	CD14$^+$	CD83$^+$	CD86$^+$
D1-1	84.5*	66.9	88.0	16.8	82.4	24.8	19.1	97.6
D1-2	98.3	80.2	82.6	17.9	76.9	4.6	2.6	98.1
D1-3	97.0	N/A	89.7	20.5	6.8	62.2	7.9	97.8
D1-4	88.5	46.4	94.1	6.0	0.3	67.5	7.0	98.7
D1-5	99.1	N/A	95.1	30.7	92.7	20.3	34.7	98.0
D1-6	85.8	29.1	60.9	3.2	6.77	4.8	13.0	92.1
D1-7	99.9	86.9	98.3	34.3	98.4	1.0	64.4	98.3
D1-8	99.2	78.3	81.8	21.0	78.4	1.7	16.1	89.3
Mean	94.0	64.6	86.3	18.8	55.3	23.4	20.6	96.2
Std. Dev.	6.58	22.47	11.81	10.70	42.63	27.08	20.24	3.51

*Values represent the percentage of cells in the DC vaccine that express the specified cell surface marker(s).

Characteristic of DC Morphology

Giemsa staining, as shown in Figure 15, revealed the standard morphology of dendritic cells which is described as a homogenous smooth intensely stained cytoplasm, cytoplasmic protrusions, a laterally positioned nucleus, and a ring of intense staining next to cell's plasma membrane. An example of the GM-CSF/IL-15 DCs

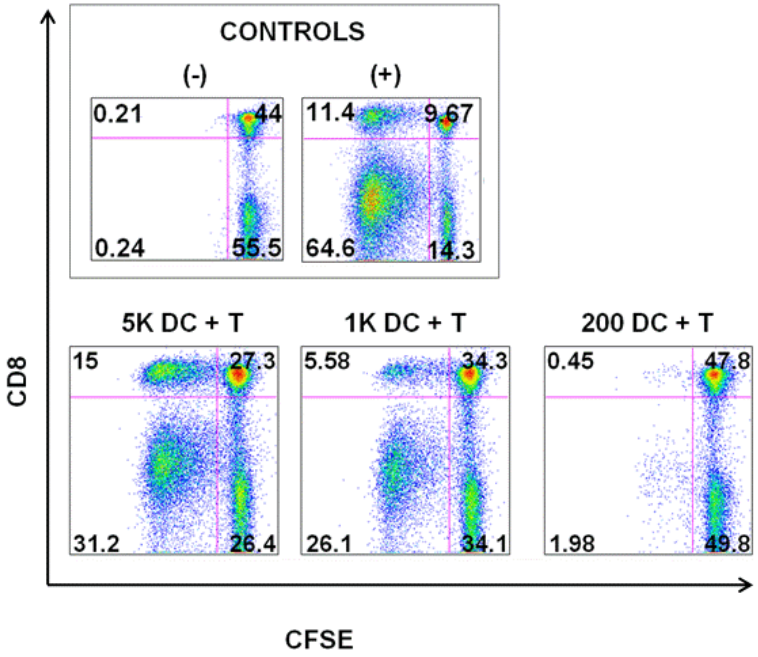
Figure 15. Morphology of monocyte-derived GM-CSF/IL-15 DC using monocytes from HIV patient D1-7.



DCs Elicit Potent Allogeneic Response

Allogeneic T cells collected from normal healthy donors were stained with CFSE. One hundred thousand lymphocytes were cultured with the DC vaccine added at various ratios from 1:20 to 1:500. After 5 days, the cells were stained with fluorescence-conjugated anti-CD3 (PerCP) and anti-CD8 (APC) and analyzed by flow cytometry. Figure 16 represents a dot plot analysis of the MLR assay performed with the DC vaccine from HIV-1 patient A3. The cells are first gated on the $CD3^+$ T cell population and further analyzed by assessing CFSE staining intensity in the $CD8^+$ and $CD8^-$ T cells. Figure 17 and Table 18 reports the allogeneic T cell responses of the $CD8^+$ population for

Figure 16. Dot plot representation of LIPO-5 loaded GM-CSF/IL-15 DC vaccine-induced allogeneic T cell proliferation. Function of LIPO-5-loaded DC vaccine from HIV-1 patient D1-7 in allogeneic MLR as measured by CFSE dilution.



HIV-1 patient DC vaccines (D1-1-D1-8). Figure 18 and Table 19 reports the allogeneic T cell responses of the $CD4^+$ population.

At a ratio of 1:20, a mean of 18.2% of $CD8^+$ T cells dilute CFSE (range, 10.9-30.1%). At a ratio of 1:100, 9.8% of $CD8^+$ T cells diluted CFSE (range, 5-4-26.5%). At a ratio of 1:500, 2.6% of $CD8^+$ T cells diluted CFSE (range, 0.4-10.3%). The proliferation background (C-) was 0.2%. CD3/CD28 dynal beads were used as a positive control, and 11.4% of $CD8^+$ T cells diluted CFSE. At a ratio of 1:20, a mean of 42.9% of $CD4^+$ T cells dilute CFSE (range, 31.2-61.9%). At a ratio of 1:100, 29.8% of $CD4^+$ T cells diluted CFSE (range, 10.3-51.9%). At a ratio of 1:500, 6.4% of $CD4^+$ T cells diluted CFSE (range, 0.5-20.4%). The proliferation background (C-) was 0.2%. CD3/CD28 dynal beads were used as a positive control, and 64.6% of $CD4^+$ T cells diluted CFSE.

Thus, monocyte-derived GM-CSF/IL-15, ANRS HIV-LIPO-5 loaded, LPS-activated DC vaccines generated from HIV-1 patients act as potent antigen presenting cells in the allogeneic MLR.

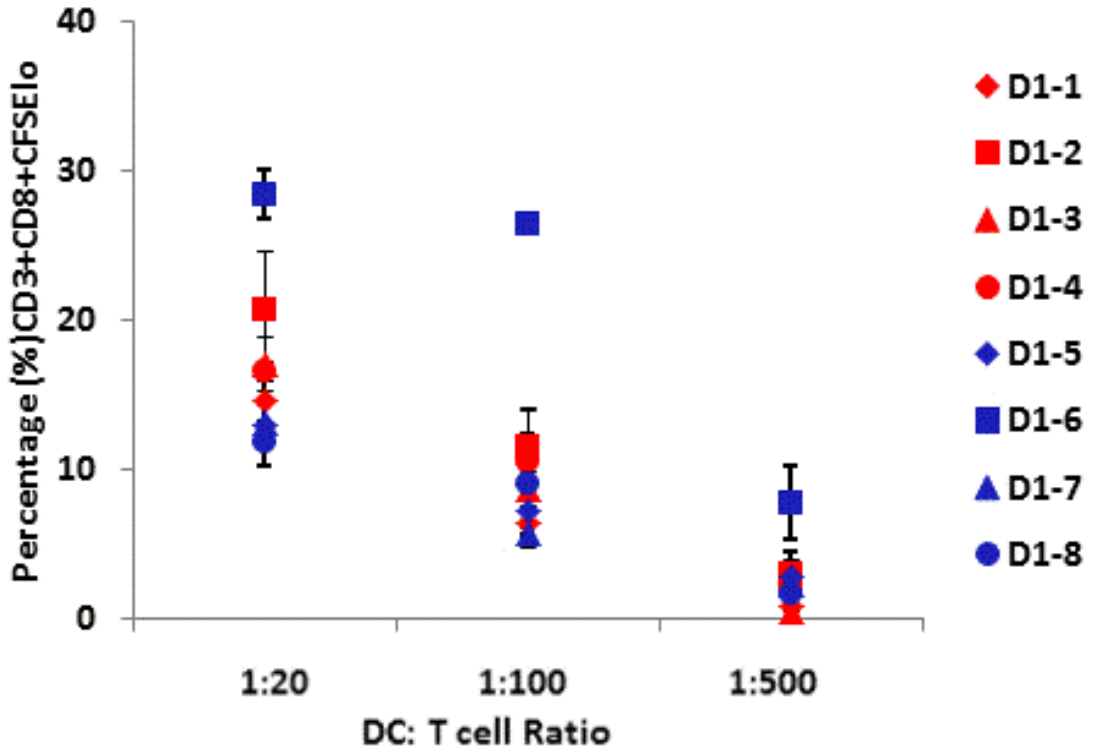


Figure 17. Comparison of LIPO-5 loaded GM-CSF/IL-15 DC vaccine-induced allogeneic $CD8^+$ T cell proliferation

Table 18. Summary of allogeneic MLR CD8 T cell proliferation by CFSE dilution induced by LIPO-5 loaded monocyte-derived GM-CSF/IL-15 DC vaccines from HIV patients.

DC Vaccine	1:20	1:100	1:500
D1-1	16.1*	5.4	0.4
D1-2	24.2	9.0	2.0
D1-3	19.1	9.2	0.5
D1-4	17.1	10.5	0.6
D1-5	13.3	5.7	3.5
D1-6	30.1	26.5	10.3
D1-7	15.0	5.6	0.5
D1-8	10.9	6.3	2.9
Mean	18.2	9.8	2.6
Std. Dev.	6.23	7.03	3.34

*Values are presented as percentage (%) $CFSE^{lo}CD3^+CD8^+$T cells

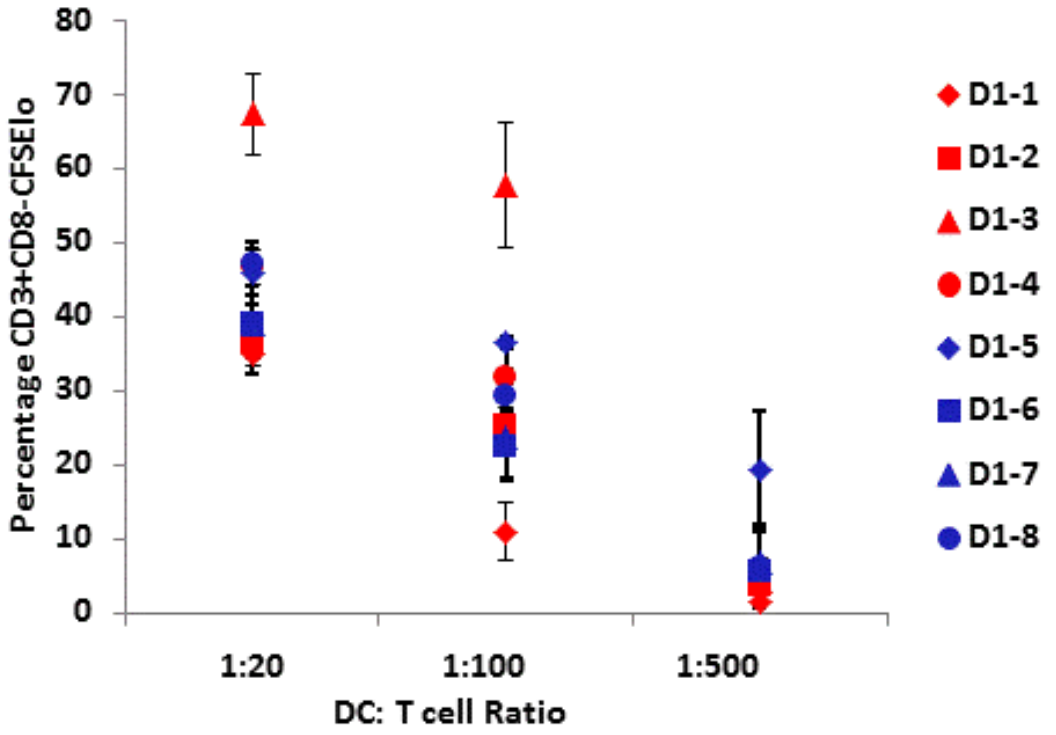


Figure 18. Comparison of LIPO-5 loaded GM-CSF/IL-15 DC vaccine-induced allogeneic $CD4^+$ T cell proliferation.

Table 19. Summary of allogeneic MLR $CD4^+$ T cell proliferation by CFSE dilution induced by LIPO-5 loaded monocyte-derived GM-CSF/IL-15 DC vaccines from HIV patients.

DC Vaccine	1:20	1:100	1:500
D1-1	34.5*	10.3	0.5
D1-2	39.6	23.9	4.1
D1-3	61.9	51.9	1.9
D1-4	49.5	31.5	3.3
D1-5	42.8	35.8	20.4
D1-6	36.1	22.6	6.7
D1-7	31.2	26.1	2.0
D1-8	47.3	36.6	12.1
Mean	42.9	29.8	6.4
Std. Dev.	9.92	12.27	6.73

*Values are presented as percentage (%) $CFSE^{lo}CD3^+CD4^+$ T cells.

IL-15 DC Elicit HIV-Specific T Cell Responses

We evaluated the capacity of the GM-CSF/IL-15 DC vaccines loaded with ANRS LIPO-5 peptides for the ability to elicit HIV-1-antigen-specific T cell responses. Initially, the DCs were used at a ratio of 1:20, which is DC to T cells, similar to the methods in chapter three. However, we did not see responses. Hypothesizing that the DCs were over-stimulating the T cells, we decided to titrate the DCs to a lower ratio of DC to T cells. We found that a ratio of 1:200 (DC: T cells) to be effective at determining the quality of antigen-specific responses (data not shown).

Ten thousand LIPO-5 (0.1 μM)-loaded DC were cultured with $2x10^6$ autologous patient lymphocytes for 10 days in the presence of IL-7 and IL-2. The expanded cells were then restimulated for 4 hours with the five individual HIV-1 peptides (10 μM, no lipidated tail) to assess the percentage of IFN-γ producing T cells by flow cytometry. A fraction of the expanded T cells were cultured without peptides to assess background IFN-γ secretion or with PMA/IONO to assess potential IFN-γ secretion and the validity of the assay.

Comprehensive scatter-graphs and statistical analysis of the antigen-specificity analysis are shown in Figure 19. The analysis began with gating on the viable lymphocyte population followed by gating on $CD3^+$ T cells. Further analysis of the gated $CD3^+$ T cells reveals that the specific HIV-1-antigen peptides are recognized by the responding IFN-γ-producing $CD8^+$ or $CD4^+$ T cells. A representative dot plot analysis of single samples is illustrated in Figure 19A. The background IFN-γ-secreting $CD8^+$ and $CD4^+$ T cells ranged from 0.02-0.09% and 0.04-0.17%, respectively. IFN-γ secretion was seen when cells were cultured with PMA/IONO. The IFN-γ-secretion of $CD8^+$ and

$CD4^+$ T cells when restimulated with PMA/IONO ranged from 5.6-41.9% and 4.8-24.3%, respectively. Figure 19B reports the comprehensive analysis of the average percentage of $CD3^+CD8^+IFN\text{-}\gamma^+$ and $CD3^+CD8^-IFN\text{-}\gamma^+$ for patients D1-1-D1-8. Positive peptide responses are identified as those greater than three standard deviations from the mean of negative controls. $CD8^+$ positive peptides had to be greater than 0.18% and $CD4^+$ positive peptides had to be greater than 0.33%.

Patient D1-1 DCs displayed $CD8^+$ T cell responses to Nef 116 and Pol 325 and $CD4^+$ T cell responses to Pol 325. Patient D1-2 DCs displayed $CD8^+$ T cell responses to Gag 253, Nef 66, Nef 116 and Pol 325 but no responses by $CD4^+$ T cells. Patient D1-3 DCs displayed $CD8^+$ T cell responses to Gag 253, Nef 66, and Pol 325 but no responses by $CD4^+$ T cells. Patient D1-4 DCs displayed $CD8^+$ T cell responses to Gag 17, Gag 253, Nef 116 and Pol 325 but no responses by $CD4^+$ T cells. Patient D1-5 DCs displayed $CD8^+$ T cell responses to Pol 325 but no responses by $CD4^+$ T cells. Patient D1-6 DCs displayed $CD8^+$ T cell responses to Nef 116 but no responses by $CD4^+$ T cells. Patient D1-7 DCs displayed $CD8^+$ T cell responses to Nef 66, Nef 116, and Pol 325 and $CD4^+$ T cell responses to Nef 66 and Nef 116. Patient D1-8 DCs displayed $CD8^+$ T cell responses to Nef 116 and Pol 325 and $CD4^+$ T cell responses Pol 325.

Interestingly, Nef 116 and Pol 325 were the most frequent $CD8^+$ T cell responses, as seen in either 6 or 7 (respectively) of the 8 patients GM-CSF/IL-15 DC vaccines based on IFN-γ secretion. Of the 8 patients, there were 24 HIV-specific T cell responses 20 of which were $CD8^+$ and 4 of which were $CD4^+$. This assay demonstrated that $CD8^+$ T cell responses to the different HIV-1 antigens in the vaccine could be elicited by all of the

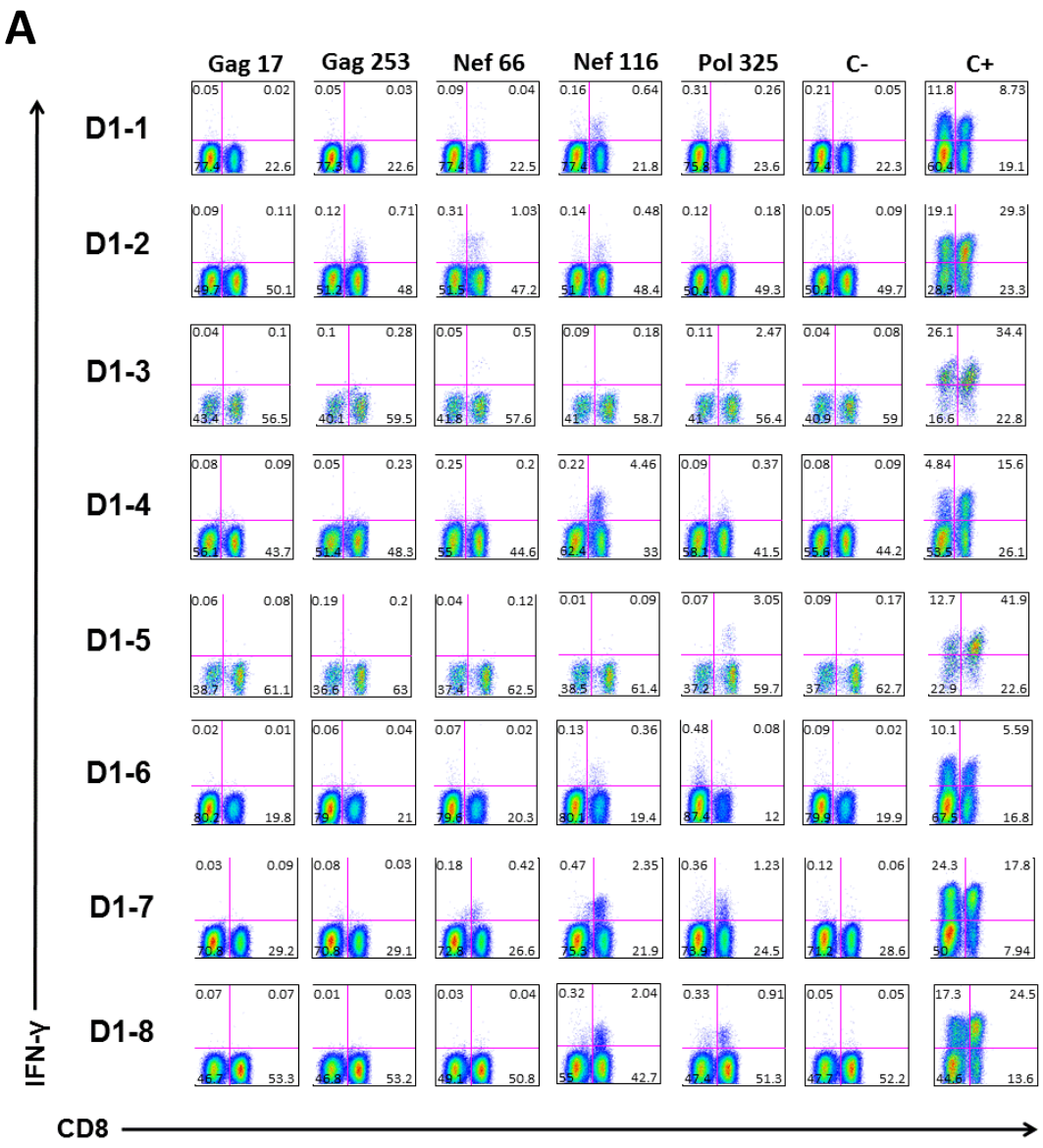


Figure 19. Compilation of IFN-γ antigen-specific T cell elicited by LIPO-5-loaded GM-CSF/IL-15 DC vaccine. ANRS HIV-LIPO-5-loaded DC vaccines induce autologous T cells to respond to specific HIV-1-antigen peptides. FACS analysis was performed to determine the percentage of DC-vaccine-expanded autologous T cells that respond to the specific HIV-1-antigen peptides in LIPO-5 ($Gag_{17\text{-}35}$ [Gag 17], $Gag_{253\text{-}284}$ [Gag 253], $Nef_{66\text{-}97}$ [Nef 66], $Nef_{116\text{-}145}$ [Nef 116] and $Pol_{325\text{-}355}$ [Pol 325]). Panel A, $CD8^+$ and $CD8^-$ T cells that produced IFN-γ were identified as responding to HIV-1-antigen peptides. The analysis was conducted with DC vaccine prepared from six HIV-1 patients (D1-1-D1-8).

B

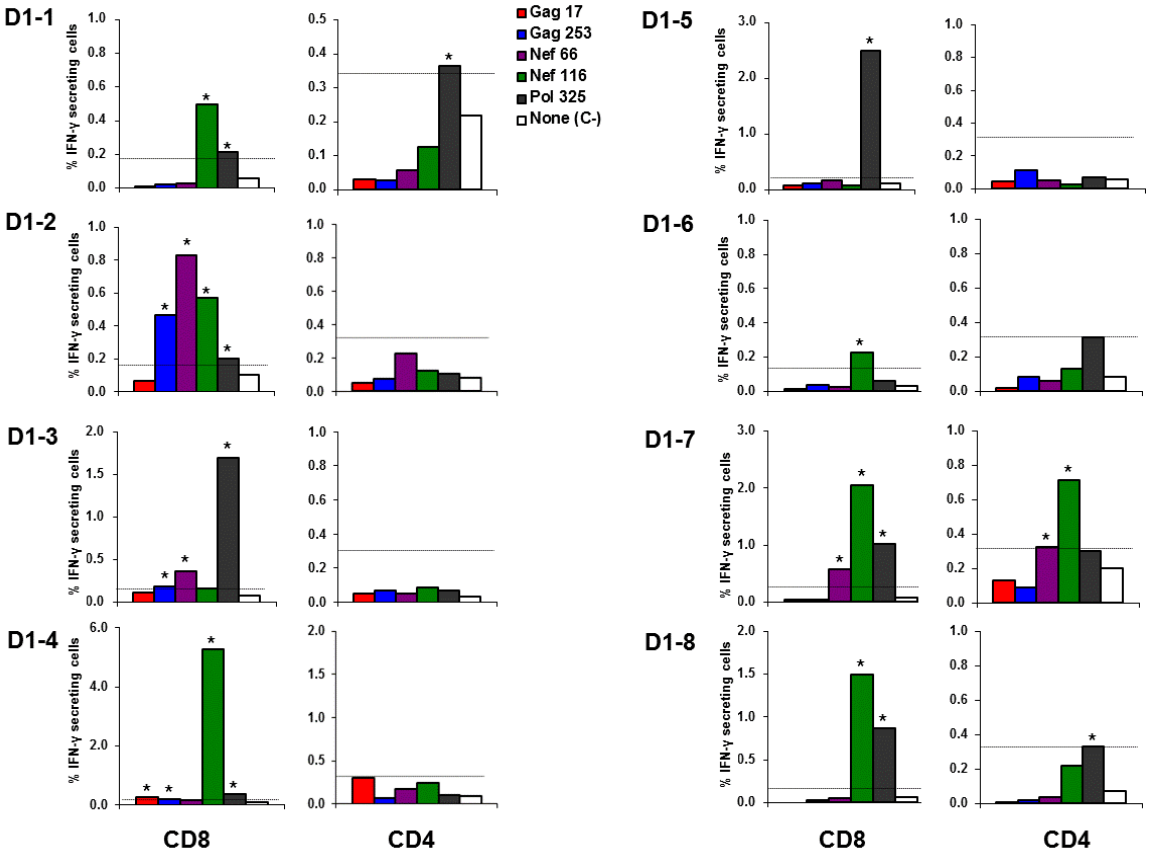


Figure 19. Compilation of IFN-γ antigen-specific T cell elicited by LIPO-5-loaded GM-CSF/IL-15 DC vaccine. ANRS HIV-LIPO-5-loaded DC vaccines induce autologous T cells to respond to specific HIV-1-antigen peptides. FACS analysis was performed to determine the percentage of DC-vaccine-expanded autologous T cells that respond to the specific HIV-1-antigen peptides in LIPO-5 ($Gag_{17\text{-}35}$ [Gag 17], $Gag_{253\text{-}284}$ [Gag 253], $Nef_{66\text{-}97}$ [Nef 66], $Nef_{116\text{-}145}$ [Nef 116] and $Pol_{325\text{-}355}$ [Pol 325]). Panel B reports the average percentage of $CD3^+CD8^+IFN\text{-}\gamma^+$ and $CD3^+CD8^-IFN\text{-}\gamma^+$ for patients D1-1-D1-8. Positive peptide responses are identified as those greater than three standard deviations from the mean of the negative controls.

HIV-1 antigen lipopeptides, although each patient had unique $CD8^+$ T cell repertoires and HLA haplotypes.

Discussion

In this chapter, we demonstrated the ability to manufacture GM-CSF/IL-15 DC vaccines using elutriated monocytes as precursors from 5 of the 8 HIV patients. This was the pre-clinical development of a process for cGMP manufacture of a fresh autologous

DC vaccine derived from monocytes isolated from HAART-treated HIV-1 infected patients. The process consisted of culturing monocytes with GM-CSF and IL-15 for four days. The resulting DC were then loaded with ANRS HIV-LIPO-5 vaccine and activated with LPS. These DC were shown to express high levels of MHC Class II and co-stimulatory molecules. When tested in an in vitro immune potency assay, the DC vaccines were capable of eliciting allogeneic T cell proliferation and autologous HIV-1 antigen-specific mainly $CD8^+$ and few $CD4^+$ T cell responses, as measured by an increase in the number of IFN-γ-producing T cells.

The enrichment of the monocytes from patient apheresis products contained on average 10.2% of contaminating neutrophils. However, enriched monocytes from three patients, D1-3, D1-4 and D1-6, had an extremely high level of neutrophil contamination (range: 18.8-30.9%) as opposed to the other patients in which had low (range: 0.6-3.7%) neutrophil contamination. Because of this high contaminate of neutrophils, the DC vaccines generated did not meet the current release criteria set for the current BIIR/ANRS-HIVax-001 DC vaccine product being tested in the DALIA 1 clinical trial.

Combining the two strategies, GM-CSF/IL-15 DC and LIPO-5, has become another attractive union for inducing a broad potent effector immune response, which is the first to be tested in humans.

CHAPTER SIX

Conclusion

My dissertation summarizes the development of several therapeutic HIV DC vaccines that possess antigen-presentation cell phenotypical and morphological characteristics, are potent stimulators of allogeneic lymphocytes, and are capable of eliciting CD8+ and CD4+ T cell responses. Although current HAART therapeutic strategies drastically reduces viral load, restores $CD4^+$ T cell counts, and has decreased the AIDS-related mortality rates, an increase in HAART-related hospitalizations have been seen due to toxicity. Furthermore, the cost of HAART makes it difficult for HIV patients to maintain the treatment plan. However, studies in LTNP have taught us that polyfunctional HIV-specific $CD8^+$ T cells are imperative in order to control HIV viral load. HAART does not offer the ability to induce long term memory T cells necessary to control disease; therefore, a need for immunotherapeutic interventions are vital.

The first aim of this study was develop a DC vaccine generated with elutriated monocytes from HAART-treated, HIV patient leukapheresis products in preparation for a phase I/II clinical trial. We demonstrated that monocytes cultured with GM-CSF/IFN-α for 3 days, pulsed with ANRS-LIPO-5 peptides and activated with LPS yielded viable and functional GM-CSF/IFN-α DCs (Cobb and others 2011). The DC vaccines expressed typical DC phenotypic markers, co-stimulatory molecules, and secrete IL-12p40, all of which are vital for the T cell interaction. Furthermore, the DCs morphology reveals protrusions of the cellular membrane which enhances the ability to communicate and educate multiple surrounding immune cells. When cultured with allogeneic

lymphocytes to test the potency of the vaccines, the DCs were able to induce proliferation even in the lowest ratio of 1 DC: 500 lymphocytes. Finally, the DC vaccines were able to elicit autologous HIV-1 antigen-specific $CD8^+$ and $CD4^+$ T cell responses, as measured by an increase in the number of IFN-γ-producing T cells but not IL-10. Furthermore, the autologous T cells that were expanded by the DC vaccines were focused on the HIV-1 antigen epitopes expressed with the LIPO-5 lipopeptides.

The second aim of this study was to employ the preclinically developed DC vaccine in a phase I/II clinical trial in humans. Nineteen patients were accrued and vaccinated with 4 doses of vaccines over the course of 3 months. After vaccination, the patients were removed from HAART to observe viral load and CD4 T cell count levels. We show that the GM-CSF/IFN-α DC vaccines manufactured from the patients resembled that of which was developed based on the phenotypic markers and potency at eliciting allogeneic T cell proliferation. Furthermore, ICS analysis of the patients before and after vaccination reveals the DC vaccines elicit immunogenicity as determined by IFN-γ, TNF-α, and IL-2 expressing $CD4^+$ and $CD8^+$ T cells.

Similar to the first aim, the third aim of this study was to develop a DC vaccine generated using GM-CSF and IL-15. Monocytes were cultured with GM-CSF and IL-15 for four days, loaded with ANRS HIV-LIPO-5 vaccine and activated with LPS. These DC were also shown to express high levels of MHC Class II and co-stimulatory molecules as well as potent at eliciting allogeneic T cell proliferation. However, a unique observation seen with these DCs early in our study was the ability to induce autologous HIV-1 antigen-specific using 10 to 20-fold less DCs as compared to GM-CSF/IFN-α.

Furthermore, HIV-specific T cell responses were mainly $CD8^+$ and few $CD4^+$ T cell responses, as measured by an increase in the number of IFN-γ-producing T cells.

The monocyte isolation using elutriation does not always yield a pure population. We observed that several HIV patient monocyte fractions had contaminating lymphocytes and neutrophils. IL-15 exhibits the ability to induce T cells and NK cells to proliferate; therefore, it is possible that any contaminating lymphocytes within the monocyte fraction could expand. Neutrophils express IL-15Rα and therefore IL-15 can induce phagocytosis by the neutrophils as well as prolong the cells life (Bouchard and others 2004; Girard and others 1996; Pelletier and others 2002). Thus, in culturing GM-CSF/IL-15 DC vaccines, limiting the cellular contaminates is an absolute necessity. For this reason, we believe several DC vaccines that were generated with the cellular contaminates did not exhibit the typical DC characteristics.

Therapeutic vaccination of HIV infected patients on HAART using DC vaccines in combination with systemic IL-7 is currently being explored. IL-7 has been shown to activate latent HIV infected cells, thus exposing a 'pesky' viral reservoir. Moreover, DC vaccines as described in this dissertation, have the ability to elicit polyfunctional T cells. As seen in LTNP, this is necessary for viral control. Thus, by activating and exposing the latent reservoir using IL-7 and eliciting polyfunctional T cells by DC vaccines, these two immunotherapies could reduce the viral reservoir that has stunted success of HAART. However, thought must be given into how often and when IL-7 and DC vaccines should be given as well as what HAART combination should be used.

In conclusion, this dissertation contains data to support the rationale to pursue clinical trials in vaccinating HAART-treated, HIV infected patients with monocyte-

derived DCs pulsed with ANRS-HIV-LIPO-5, activated with LPS. Overall, the goal in therapeutic HIV DC vaccines is to convert chronic HIV infected patients into LTNP so that HAART can be eliminated. With this goal in mind, I look forward to what the future of immunotherapy has in store in combating this debilitating disease.

REFERENCES

1982. Opportunistic infections and Kaposi's sarcoma among Haitians in the United States. MMWR. Morbidity and mortality weekly report 31(26):353-4, 360-1.

1990. Interim proposal for a WHO Staging System for HIV infection and Disease. Releve epidemiologique hebdomadaire / Section d'hygiene du Secretariat de la Societe des Nations = Weekly epidemiological record / Health Section of the Secretariat of the League of Nations 65(29):221-4.

Abrams D, Levy Y, Losso MH, Babiker A, Collins G, Cooper DA, Darbyshire J, Emery S, Fox L, Gordin F et al. . 2009. Interleukin-2 therapy in patients with HIV infection. The New England journal of medicine 361(16):1548-59.

Alvarez D, Vollmann EH, von Andrian UH. 2008. Mechanisms and Consequences of Dendritic Cell Migration. Immunity 29(3):325-342.

Amirayan-Chevillard N, Tissot-Dupont H, Capo C, Brunet C, Dignat-George F, Obadia Y, Gallais H, Mege JL. 2000. Impact of highly active anti-retroviral therapy (HAART) on cytokine production and monocyte subsets in HIV-infected patients. Clinical and experimental immunology 120(1):107-12.

Appay, V., Nixon, D.F., Donahoe, S.M., Gillespie, G.M., Dong, T., King, A., Ogg, G.S. 1982. Opportunistic infections and Kaposi's sarcoma among Haitians in the United States. MMWR. Morbidity and mortality weekly report 31(26):353-4, 360-1.

Appay V, Nixon DF, Donahoe SM, Gillespie GM, Dong T, King A, Ogg GS, Spiegel HM, Conlon C, Spina CA et al. 2000. HIV-specific CD8(+) T cells produce antiviral cytokines but are impaired in cytolytic function. J. Exp. Med. 192:63-76.

Banchereau J, Palucka AK. 2005. Dendritic cells as therapeutic vaccines against cancer. Nature reviews. Immunology 5(4):296-306.

Banchereau J, Steinman RM. 1998. Dendritic cells and the control of immunity. Nature 392(6673):245-252.

Banchereau J, Ueno H, Dhodapkar M, Connolly J, Finholt JP, Klechevsky E, Blanck JP, Johnston DA, Palucka AK, Fay J. 2005. Immune and clinical outcomes in patients with stage IV melanoma vaccinated with peptide-pulsed dendritic cells derived from CD34(+) progenitors and activated with type I interferon. Journal of Immunotherapy 28(5):505-516.

Barre-Sinoussi F, Chermann JC, Rey F, Nugeyre MT, Chamaret S, Gruest J, Dauguet C, Axler-Blin C, Vezinet-Brun F, Rouzioux C et al. . 1983. Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS). Science 220(4599):868-71.

Baslund B, Tvede N, Danneskiold-Samsoe B, Larsson P, Panayi G, Petersen J, Petersen LJ, Beurskens FJ, Schuurman J, van de Winkel JG et al. . 2005. Targeting interleukin-15 in patients with rheumatoid arthritis: a proof-of-concept study. Arthritis and rheumatism 52(9):2686-92.

Bennett L, Palucka AK, Arce E, Cantrell V, Borvak J, Banchereau J, Pascual V. 2003. Interferon and granulopoiesis signatures in systemic lupus erythematosus blood. The Journal of experimental medicine 197(6):711-23.

Berson JF, Long D, Doranz BJ, Rucker J, Jirik FR, Doms RW. 1996. A seven-transmembrane domain receptor involved in fusion and entry of T cell-tropic human immunodeficiency virus type 1 strains. Journal of virology 70(9):6288-95.

Blanco P, Palucka AK, Gill M, Pascual V, Banchereau J. 2001. Induction of dendritic cell differentiation by IFN-alpha in systemic lupus erythematosus. Science 294(5546):1540-3.

Bonaldo MC, Martins MA, Rudersdorf R, Mudd PA, Sacha JB, Piaskowski SM, Neves PCC, de Santana MGV, Vojnov L, Capuano S et al. . 2010. Recombinant Yellow Fever Vaccine Virus 17D Expressing Simian Immunodeficiency Virus SIVmac239 Gag Induces SIV-Specific CD8(+) T cell Responses in Rhesus Macaques. J Virol 84(7):3699-3706.

Borrow P, Lewicki H, Hanh BH, Shaw GM, Oldstone MB. 1994. Virus-specific CD8+ cytotoxic T-lymphocyte activity associated with control of viremia in primary human immunodeficiency virus type 1 infection. J. Virol. 68:6103-6110.

Bouchard A, Ratthe C, Girard D. 2004. Interleukin-15 delays human neutrophil apoptosis by intracellular events and not via extracellular factors: role of Mcl-1 and decreased activity of caspase-3 and caspase-8. Journal of leukocyte biology 75(5):893-900.

Buchacz K, Baker RK, Moorman AC, Richardson JT, Wood KC, Holmberg SD, Brooks JT. 2008. Rates of hospitalizations and associated diagnoses in a large multisite cohort of HIV patients in the United States, 1994-2005. AIDS 22(11):1345-54.

Buchbinder SP, Mehrotra DV, Duerr A, Fitzgerald DW, Mogg R, Li D, Gilbert PB, Lama JR, Marmor M, Del Rio C et al. . 2008. Efficacy assessment of a cell-mediated immunity HIV-1 vaccine (the Step Study): a double-blind, randomised, placebo-controlled, test-of-concept trial. Lancet 372(9653):1881-93.

Cao Y, Qin L, Zhang L, Safrit J, Ho DD. 1995. Virologic and immunologic characterization of long-term survivors of human immunodeficiency virus type 1 infection. N.Engl.J.Med. 332:201-208.

Carbonneil C, Aouba A, Burgard M, Cardinaud S, Rouzioux C, Langlade-Demoyen P, Weiss L. 2003. Dendritic cells generated in the presence of granulocyte-macrophage colony-stimulating factor and IFN-alpha are potent inducers of HIV-specific CD8 T cells. AIDS 17(12):1731-40.

Carcelain G, Tubiana R, Samri A, Calvez V, Delaugerre C, Agut H, Katlama C, Autran B. 2001. Transient mobilization of human immunodeficiency virus (HIV)-specific CD4 T-helper cells fails to control virus rebounds during intermittent antiretroviral therapy in chronic HIV type 1 infection. J Virol 75(1):234-41.

Carter M. 2003. HAART patients at greater risk of severe side effects than AIDS. IAPAC monthly 9(12):314.

Castro KG, Valdiserri RO, Curran JW. 1992. Perspectives on HIV/AIDS epidemiology and prevention from the Eighth International Conference on AIDS. American journal of public health 82(11):1465-70.

Catalfamo M, Di Mascio M, Hu Z, Srinivasula S, Thaker V, Adelsberger J, Rupert A, Baseler M, Tagaya Y, Roby G et al. . 2008. HIV infection-associated immune activation occurs by two distinct pathways that differentially affect CD4 and CD8 T cells. Proceedings of the National Academy of Sciences of the United States of America 105(50):19851-6.

Catanzaro AT, Koup RA, Roederer M, Bailer RT, Enama ME, Moodie Z, Gu L, Martin JE, Novik L, Chakrabarti BK et al. . 2006. Phase 1 safety and immunogenicity evaluation of a multiclade HIV-1 candidate vaccine delivered by a replication-defective recombinant adenovirus vector. The Journal of infectious diseases 194(12):1638-49.

Champagne P, Ogg GS, King AS, Knabenhans C, Ellefsen K, Nobile M, Appay V, Rizzardi GP, Fleury S, Lipp M et al. . 2001. Skewed maturation of memory HIV-specific CD8 T lymphocytes. Nature 410:106-11.

Chermann JC, Donker G, Yahi N, Salaun D, Guettari N, Gayet O, Hirsh I. 1991. Discrepancies in AIDS virus data. Nature 351(6324):277-8.

Chun TW, Fauci AS. 1999. Latent reservoirs of HIV: obstacles to the eradication of virus. Proceedings of the National Academy of Sciences of the United States of America 96(20):10958-61.

Cobb A, Roberts LK, Palucka AK, Mead H, Montes M, Ranganathan R, Burkeholder S, Finholt JP, Blankenship D, King B et al. . 2011. Development of a HIV-1 lipopeptide antigen pulsed therapeutic dendritic cell vaccine. Journal of immunological methods 365(1-2):27-37.

Coffin J, Haase A, Levy JA, Montagnier L, Oroszlan S, Teich N, Temin H, Toyoshima K, Varmus H, Vogt P et al. . 1986. Human immunodeficiency viruses. Science 232(4751):697.

Colombetti S, Levy F, Chapatte L. 2009. IL-7 adjuvant treatment enhances long-term tumor-antigen-specific CD8+ T cell responses after immunization with recombinant lentivector. Blood 113(26):6629-37.

Connolly NC, Whiteside TL, Wilson C, Kondragunta V, Rinaldo CR, Riddler SA. 2008. Therapeutic immunization with human immunodeficiency virus type 1 (HIV-1) peptide-loaded dendritic cells is safe and induces immunogenicity in HIV-1-infected individuals. Clinical and vaccine immunology : CVI 15(2):284-92.

Cooper DA, Gold J, Maclean P, Donovan B, Finlayson R, Barnes TG, Michelmore HM, Brooke P, Penny R. 1985. Acute AIDS retrovirus infection. Definition of a clinical illness associated with seroconversion. Lancet 1(8428):537-40.

Cooper DA, Tindall B, Wilson EJ, Imrie AA, Penny R. 1988. Characterization of T lymphocyte responses during primary infection with human immunodeficiency virus. The Journal of infectious diseases 157(5):889-96.

Currier JR, Ngauy V, de Souza MS, Ratto-Kim S, Cox JH, Polonis VR, Earl P, Moss B, Peel S, Slike B et al. . 2010. Phase I safety and immunogenicity evaluation of MVA-CMDR, a multigenic, recombinant modified vaccinia Ankara-HIV-1 vaccine candidate. PloS one 5(11):e13983.

Dalgleish AG, Beverley PC, Clapham PR, Crawford DH, Greaves MF, Weiss RA. 1984. The CD4 (T4) antigen is an essential component of the receptor for the AIDS retrovirus. Nature 312(5996):763-7.

Dall'era MC, Cardarelli PM, Preston BT, Witte A, Davis JC, Jr. 2005. Type I interferon correlates with serological and clinical manifestations of SLE. Ann Rheum Dis 64(12):1692-7.

Deeks SG, Walker BD. 2007. Human immunodeficiency virus controllers: mechanisms of durable virus control in the absence of antiretroviral therapy. Immunity 27(3):406-16.

Della Bella S, Nicola S, Riva A, Biasin M, Clerici M, Villa ML. 2004. Functional repertoire of dendritic cells generated in granulocyte macrophage-colony stimulating factor and interferon-alpha. Journal of leukocyte biology 75(1):106-16.

Doranz BJ, Rucker J, Yi Y, Smyth RJ, Samson M, Peiper SC, Parmentier M, Collman RG, Doms RW. 1996. A dual-tropic primary HIV-1 isolate that uses fusin and the beta-chemokine receptors CKR-5, CKR-3, and CKR-2b as fusion cofactors. Cell 85(7):1149-58.

Dubsky P, Saito H, Leogier M, Dantin C, Connolly JE, Banchereau J, Palucka AK. 2007. IL-15-induced human DC efficiently prime melanoma-specific naive CD8+ T cells to differentiate into CTL. European journal of immunology 37(6):1678-90.

Dubsky P, Ueno H, Piqueras B, Connolly J, Banchereau J, Palucka AK. 2005. Human dendritic cell subsets for vaccination. Journal of clinical immunology 25(6):551-72.

Durier C, Launay O, Meiffredy V, Saidi Y, Salmon D, Levy Y, Guillet JG, Pialoux G, Aboulker JP. 2006. Clinical safety of HIV lipopeptides used as vaccines in healthy volunteers and HIV-infected adults. AIDS 20(7):1039-49.

Dybul M, Fauci AS, Bartlett JG, Kaplan JE, Pau AK. 2002. Guidelines for using antiretroviral agents among HIV-infected adults and adolescents. Annals of internal medicine 137(5 Pt 2):381-433.

Earl PL, Doms RW, Moss B. 1990. Oligomeric structure of the human immunodeficiency virus type 1 envelope glycoprotein. Proceedings of the National Academy of Sciences of the United States of America 87(2):648-52.

Feng X, Wu H, Grossman JM, Hanvivadhanakul P, FitzGerald JD, Park GS, Dong X, Chen W, Kim MH, Weng HH et al. . 2006. Association of increased interferon-inducible gene expression with disease activity and lupus nephritis in patients with systemic lupus erythematosus. Arthritis Rheum 54(9):2951-62.

Fry TJ, Mackall CL. 2002a. Interleukin-7 and immunorestoration in HIV: beyond the thymus. Journal of hematotherapy & stem cell research 11(5):803-7.

Fry TJ, Mackall CL. 2002b. Interleukin-7: from bench to clinic. Blood 99(11):3892-904.

Gallo RC, Salahuddin SZ, Popovic M, Shearer GM, Kaplan M, Haynes BF, Palker TJ, Redfield R, Oleske J, Safai B et al. . 1984. Frequent detection and isolation of cytopathic retroviruses (HTLV-III) from patients with AIDS and at risk for AIDS. Science 224(4648):500-3.

Gelderblom HR, Ozel M, Pauli G. 1989. Morphogenesis and morphology of HIV. Structure-function relations. Archives of virology 106(1-2):1-13.

Girard D, Paquet ME, Paquin R, Beaulieu AD. 1996. Differential effects of interleukin-15 (IL-15) and IL-2 on human neutrophils: modulation of phagocytosis, cytoskeleton rearrangement, gene expression, and apoptosis by IL-15. Blood 88(8):3176-84.

Goepfert PA, Bansal A, Edwards BH, Ritter GD, Jr., Tellez I, McPherson SA, Sabbaj S, Mulligan MJ. 2000. A significant number of human immunodeficiency virus epitope-specific cytotoxic T lymphocytes detected by tetramer binding do not produce gamma interferon. J Virol 74(21):10249-55.

Gottlieb MS. 2006. Pneumocystis pneumonia--Los Angeles. 1981. American journal of public health 96(6):980-1; discussion 982-3.

Gottlieb MS, Schroff R, Schanker HM, Weisman JD, Fan PT, Wolf RA, Saxon A. 1981. Pneumocystis carinii pneumonia and mucosal candidiasis in previously healthy homosexual men: evidence of a new acquired cellular immunodeficiency. The New England journal of medicine 305(24):1425-31.

Goujard C, Marcellin F, Hendel-Chavez H, Burgard M, Meiffredy V, Venet A, Rouzioux C, Taoufik Y, El Habib R, Beumont-Mauviel M et al. . 2007. Interruption of antiretroviral therapy initiated during primary HIV-1 infection: Impact of a therapeutic vaccination strategy combined with interleukin (IL)-2 compared with IL-2 alone in the ANRS 095 randomized study. Aids Research and Human Retroviruses 23(9):1105-1113.

Groopman JE. 1990. Zidovudine intolerance. Reviews of infectious diseases 12 Suppl 5:S500-6.

Guo HG, Chermann JC, Waters D, Hall L, Louie A, Gallo RC, Streicher H, Reitz MS, Popovic M, Blattner W. 1991. Sequence analysis of original HIV-1. Nature 349(6312):745-6.

Hill AM, Smith C. 2007. Analysis of treatment costs for HIV RNA reductions and CD4 increases for darunavir versus other antiretrovirals in treatment-experienced, HIV-infected patients. HIV clinical trials 8(3):121-31.

Homsy J, Meyer M, Tateno M, Clarkson S, Levy JA. 1989. The Fc and not CD4 receptor mediates antibody enhancement of HIV infection in human cells. Science 244(4910):1357-60.

Hosmalin A, Andrieu M, Loing E, Desoutter JF, Hanau D, Gras-Masse H, Dautry-Varsat A, Guillet JG. 2001. Lipopeptide presentation pathway in dendritic cells. Immunology Letters 79(1-2):97-100.

Janeway C. 2005. Immunobiology : the immune system in health and disease. New York: Garland Science.

Kaufmann GR, Zaunders JJ, Cunningham P, Kelleher AD, Grey P, Smith D, Carr A, Cooper DA. 2000. Rapid restoration of CD4 T cell subsets in subjects receiving antiretroviral therapy during primary HIV-1 infection. Aids 14(17):2643-2651.

Kirou KA, Lee C, George S, Louca K, Peterson MG, Crow MK. 2005. Activation of the interferon-alpha pathway identifies a subgroup of systemic lupus erythematosus patients with distinct serologic features and active disease. Arthritis Rheum 52(5):1491-503.

Klatzmann D, Barre-Sinoussi F, Nugeyre MT, Danquet C, Vilmer E, Griscelli C, Brun-Veziret F, Rouzioux C, Gluckman JC, Chermann JC et al. . 1984a. Selective tropism of lymphadenopathy associated virus (LAV) for helper-inducer T lymphocytes. Science 225(4657):59-63.

Klatzmann D, Champagne E, Chamaret S, Gruest J, Guetard D, Hercend T, Gluckman JC, Montagnier L. 1984b. T-lymphocyte T4 molecule behaves as the receptor for human retrovirus LAV. Nature 312(5996):767-8.

Koup RA, Safrit JT, Yunzhen C, Andrews CA, McLeod G, Borkowsky W, Farthing C, Ho DD. 1994. Temporal association of cellular immune responses with the initial control of viremia in primary human immunodeficiency virus type 1 syndrome. J. Virol. 68:4650-4655.

Kovacs JA, Imamichi H, Vogel S, Metcalf JA, Dewar RL, Baseler M, Stevens R, Adelsberger J, Lambert L, Davey RT, Jr. et al. . 2000. Effects of intermittent interleukin-2 therapy on plasma and tissue human immunodeficiency virus levels and quasi-species expression. The Journal of infectious diseases 182(4):1063-9.

Lang W, Perkins H, Anderson RE, Royce R, Jewell N, Winkelstein W, Jr. 1989. Patterns of T lymphocyte changes with human immunodeficiency virus infection: from seroconversion to the development of AIDS. Journal of acquired immune deficiency syndromes 2(1):63-9.

Lapenta C, Santini SM, Logozzi M, Spada M, Andreotti M, Di Pucchio T, Parlato S, Belardelli F. 2003. Potent immune response against HIV-1 and protection from virus challenge in hu-PBL-SCID mice immunized with inactivated virus-pulsed dendritic cells generated in the presence of IFN-alpha. The Journal of experimental medicine 198(2):361-7.

Lapenta C, Santini SM, Spada M, Donati S, Urbani F, Accapezzato D, Franceschini D, Andreotti M, Barnaba V, Belardelli F. 2006. IFN-alpha-conditioned dendritic cells are highly efficient in inducing cross-priming CD8(+) T cells against exogenous viral antigens. European journal of immunology 36(8):2046-60.

Launay O, Durier C, Desaint C, Silbermann B, Jackson A, Pialoux G, Bonnet B, Poizot-Martin I, Gonzalez-Canali G, Cuzin L et al. . 2007. Cellular Immune Responses Induced with Dose-Sparing Intradermal Administration of HIV Vaccine to HIV-Uninfected Volunteers in the ANRS VAC16 Trial. Plos One 2(8):-.

Laurence J, Saunders A, Early E, Salmon JE. 1990. Human immunodeficiency virus infection of monocytes: relationship to Fc-gamma receptors and antibody-dependent viral enhancement. Immunology 70(3):338-43.

Levy JA. 1994. HIV and the pathogenesis of AIDS. Washington, D.C.: ASM Press.

Levy JA, Hoffman AD, Kramer SM, Landis JA, Shimabukuro JM, Oshiro LS. 1984. Isolation of lymphocytopathic retroviruses from San Francisco patients with AIDS. Science 225(4664):840-2.

Levy Y, Gahery-Segard H, Durier C, Lascaux AS, Goujard C, Meiffredy V, Rouzioux C, El Habib R, Beumont-Mauviel M, Guillet JG et al. . 2005. Immunological and virological efficacy of a therapeutic immunization combined with interleukin-2 in chronically HIV-1 infected patients. AIDS 19(3):279-86.

Levy Y. 2006. Cytokine-based modulation of immune function in HIV infection. Current opinion in HIV and AIDS 1(1):69-73.

Levy Y, Lacabaratz C, Weiss L, Viard JP, Goujard C, Lelievre JD, Boue F, Molina JM, Rouzioux C, Avettand-Fenoel V et al. . 2009. Enhanced T cell recovery in HIV-1-infected adults through IL-7 treatment. The Journal of clinical investigation 119(4):997-1007.

Liu JY, O'Brien KL, Lynch DM, Simmons NL, La Porte A, Riggs AM, Abbink P, Coffey RT, Grandpre LE, Seaman MS et al. . 2009. Immune control of an SIV challenge by a T cell-based vaccine in rhesus monkeys. Nature 457(7225):87-91.

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

Lu YL, Spearman P, Ratner L. 1993. Human immunodeficiency virus type 1 viral protein R localization in infected cells and virions. Journal of virology 67(11):6542-50.

Margolick JB, Munoz A, Donnenberg AD, Park LP, Galai N, Giorgi JV, O'Gorman MR, Ferbas J. 1995. Failure of T cell homeostasis preceding AIDS in HIV-1 infection. The Multicenter AIDS Cohort Study. Nature medicine 1(7):674-80.

Martinon F, Grasmasse H, Boutillon C, Chirat F, Deprez B, Guillet JG, Gomard E, Tartar A, Levy JP. 1992. Immunization of Mice with Lipopeptides Bypasses the Prerequisite for Adjuvant - Immune-Response of Balb/C Mice to Human-Immunodeficiency-Virus Envelope Glycoprotein. Journal of Immunology 149(10):3416-3422.

McMichael AJ, Borrow P, Tomaras GD, Goonetilleke N, Haynes BF. 2010. The immune response during acute HIV-1 infection: clues for vaccine development. Nature Reviews Immunology 10(1):11-23.

Melief CJM. 2008. Cancer immunotherapy by dendritic cells. Immunity 29(3):372-383.

Migueles SA, Osborne CM, Royce C, Compton AA, Joshi RP, Weeks KA, Rood JE, Berkley AM, Sacha JB, Cogliano-Shutta NA et al. . 2008. Lytic granule loading of CD8+ T cells is required for HIV-infected cell elimination associated with immune control. Immunity 29(6):1009-21.

Millogo A, Lankoande D, Yameogo I, Yameogo AA, Sawadogo AB. 2008. [Polyneuropathies in patients treated with HAART in Bobo-Dioulasso hospital, Burkina Faso]. Bulletin de la Societe de pathologie exotique 101(1):11-3.

Montagnier L, Chermann JC, Barre-Sinoussi F, Klatzmann D, Wain-Hobson S, Alizon M, Clavel F, Brun-Vezinet F, Vilmer E, Rouzioux C et al. . 1984. Lymphadenopathy associated virus and its etiological role in AIDS. Princess Takamatsu symposia 15:319-31.

Mosmann TR, Sad S. 1996. The expanding universe of T cell subsets: Th1, Th2 and more. Immunology today 17(3):138-46.

Mueller YM, Makar V, Bojczuk PM, Witek J, Katsikis PD. 2003. IL-15 enhances the function and inhibits CD95/Fas-induced apoptosis of human CD4+ and CD8+ effector-memory T cells. International immunology 15(1):49-58.

Nahmias AJ, Weiss J, Yao X, Lee F, Kodsi R, Schanfield M, Matthews T, Bolognesi D, Durack D, Motulsky A et al. . 1986. Evidence for human infection with an HTLV III/LAV-like virus in Central Africa, 1959. Lancet 1(8492):1279-80.

Niewold TB, Hua J, Lehman TJ, Harley JB, Crow MK. 2007. High serum IFN-alpha activity is a heritable risk factor for systemic lupus erythematosus. Genes Immun 8(6):492-502.

Ozel M, Pauli G, Gelderblom HR. 1988. The organization of the envelope projections on the surface of HIV. Archives of virology 100(3-4):255-66.

Palucka AK, Ueno H, Fay JW, Banchereau J. 2007. Taming cancer by inducing immunity via dendritic cells. Immunological reviews 220:129-50.

Pantaleo G, Walker BD. 2001. Retroviral immunology : immune response and restoration. Totowa, N.J.: Humana Press.

Pelletier M, Ratthe C, Girard D. 2002. Mechanisms involved in interleukin-15-induced suppression of human neutrophil apoptosis: role of the anti-apoptotic Mcl-1 protein and several kinases including Janus kinase-2, p38 mitogen-activated protein kinase and extracellular signal-regulated kinases-1/2. FEBS letters 532(1-2):164-70.

Peters BS, Jaoko W, Vardas E, Panayotakopoulos G, Fast P, Schmidt C, Gilmour J, Bogoshi M, Omosa-Manyonyi G, Dally L et al. . 2007. Studies of a prophylactic HIV-1 vaccine candidate based on modified vaccinia virus Ankara (MVA) with and without DNA priming: effects of dosage and route on safety and immunogenicity. Vaccine 25(11):2120-7.

Pett SL. 2009. Immunotherapies in HIV-1 infection. Current opinion in HIV and AIDS 4(3):188-93.

Pett SL, Kelleher AD, Emery S. 2010. Role of interleukin-2 in patients with HIV infection. Drugs 70(9):1115-30.

Pialoux G, Gahery-Segard H, Sermet S, Poncelet H, Fournier S, Gerard L, Tartar A, Gras-Masse H, Levy JP, Guillet JG et al. . 2001. Lipopeptides induce cell-mediated anti-HIV immune responses in seronegative volunteers. Aids 15(10):1239-1249.

Pialoux G, Quercia RP, Gahery H, Daniel N, Slama L, Girard PM, Bonnard P, Rozenbaum W, Schneider V, Salmon D et al. . 2008. Immunological responses and long-term treatment interruption after human immunodeficiency virus type 1 (HIV-1) lipopeptide immunization of HIV-1-infected patients: the LIPTHERA study. Clinical and Vaccine Immunology 15(3):562-568.

Platanias LC. 2005. Mechanisms of type-I- and type-II-interferon-mediated signalling. Nature reviews. Immunology 5(5):375-86.

Popovic M, Sarngadharan MG, Read E, Gallo RC. 1984. Detection, isolation, and continuous production of cytopathic retroviruses (HTLV-III) from patients with AIDS and pre-AIDS. Science 224(4648):497-500.

Priddy FH, Brown D, Kublin J, Monahan K, Wright DP, Lalezari J, Santiago S, Marmor M, Lally M, Novak RM et al. . 2008. Safety and immunogenicity of a replication-incompetent adenovirus type 5 HIV-1 clade B gag/pol/nef vaccine in healthy adults. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America 46(11):1769-81.

Pulliam L, Gascon R, Stubblebine M, McGuire D, McGrath MS. 1997. Unique monocyte subset in patients with AIDS dementia. Lancet 349(9053):692-5.

Ratner L, Gallo RC, Wong-Staal F. 1985. HTLV-III, LAV, ARV are variants of same AIDS virus. Nature 313(6004):636-7.

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

Routy JP, Boulassel MR, Yassine-Diab B, Nicolette C, Healey D, Jain R, Landry C, Yegorov O, Tcherepanova I, Monesmith T et al. . 2010. Immunologic activity and safety of autologous HIV RNA-electroporated dendritic cells in HIV-1 infected patients receiving antiretroviral therapy. Clinical Immunology 134(2):140-147.

Rozis G, Benlahrech A, Duraisingham S, Gotch F, Patterson S. 2008. Human Langerhans' cells and dermal-type dendritic cells generated from CD34 stem cells express different toll-like receptors and secrete different cytokines in response to toll-like receptor ligands. Immunology 124(3):329-38.

Rucker J, Samson M, Doranz BJ, Libert F, Berson JF, Yi Y, Smyth RJ, Collman RG, Broder CC, Vassart G et al. . 1996. Regions in beta-chemokine receptors CCR5 and CCR2b that determine HIV-1 cofactor specificity. Cell 87(3):437-46.

Sabado RL, Bhardwaj N. 2010. Directing dendritic cell immunotherapy towards successful cancer treatment. Immunotherapy 2(1):37-56.

Sallusto F, Lanzavecchia A. 1994. Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor alpha. The Journal of experimental medicine 179(4):1109-18.

Salmon-Ceron D, Durier C, Desaint C, Cuzin L, Surenaud M, Ben Hamouda N, Lelievre JD, Bonnet B, Pialoux G, Poizot-Martin I et al. . 2010. Immunogenicity and safety of an HIV-1 lipopeptide vaccine in healthy adults: a phase 2 placebo-controlled ANRS trial. Aids 24(14):2211-2223.

Shen L, Siliciano RF. 2008. Viral reservoirs, residual viremia, and the potential of highly active antiretroviral therapy to eradicate HIV infection. The Journal of allergy and clinical immunology 122(1):22-8.

Soriano V, Puoti M, Garcia-Gasco P, Rockstroh JK, Benhamou Y, Barreiro P, McGovern B. 2008. Antiretroviral drugs and liver injury. AIDS 22(1):1-13.

Spiegel, H.M., Conlon, C., Spina, C.A., Havlir, D.V., Richman, D.D., Waters, A., Easterbrook, P., McMichael, A.J. and Rowland-Jones, S.L., 2000, HIV-specific CD8(+) T cells produce antiviral cytokines but are impaired in cytolytic function. J. Exp. Med. 192, 63-76.

Steel JC, Ramlogan CA, Yu P, Sakai Y, Forni G, Waldmann TA, Morris JC. 2010. Interleukin-15 and its receptor augment dendritic cell vaccination against the neu oncogene through the induction of antibodies partially independent of CD4 help. Cancer research 70(3):1072-81.

Steinman RM, Banchereau J. 2007. Taking dendritic cells into medicine. Nature 449(7161):419-26.

Takeda A, Tuazon CU, Ennis FA. 1988. Antibody-enhanced infection by HIV-1 via Fc receptor-mediated entry. Science 242(4878):580-3.

Timmerman JM, Levy R. 2000. Linkage of foreign carrier protein to a self-tumor antigen enhances the immunogenicity of a pulsed dendritic cell vaccine. Journal of Immunology 164(9):4797-4803.

Tindall B, Barker S, Donovan B, Barnes T, Roberts J, Kronenberg C, Gold J, Penny R, Cooper D. 1988. Characterization of the acute clinical illness associated with human immunodeficiency virus infection. Archives of internal medicine 148(4):945-9.

Thieblemont N, Weiss L, Sadeghi HM, Estcourt C, Haeffner-Cavaillon N. 1995. CD14lowCD16high: a cytokine-producing monocyte subset which expands during human immunodeficiency virus infection. European journal of immunology 25(12):3418-24.

Trkola A, Dragic T, Arthos J, Binley JM, Olson WC, Allaway GP, Cheng-Mayer C, Robinson J, Maddon PJ, Moore JP. 1996. CD4-dependent, antibody-sensitive interactions between HIV-1 and its co-receptor CCR-5. Nature 384(6605):184-7.

Ueno H, Klechevsky E, Morita R, Aspord C, Cao T, Matsui T, Di Pucchio T, Connolly J, Fay JW, Pascual V et al. . 2007. Dendritic cell subsets in health and disease. Immunological reviews 219:118-42.

Vignali DAA, Collison LW, Workman CJ. 2008. How regulatory T cells work. Nature Reviews Immunology 8(7):523-532.

Virgin HW, Walker BD. 2010. Immunology and the elusive AIDS vaccine. Nature 464(7286):224-231.

Waldmann TA. 2006. The biology of interleukin-2 and interleukin-15: implications for cancer therapy and vaccine design. Nature reviews. Immunology 6(8):595-601.

Wang FX, Xu Y, Sullivan J, Souder E, Argyris EG, Acheampong EA, Fisher J, Sierra M, Thomson MM, Najera R et al. . 2005. IL-7 is a potent and proviral strain-specific inducer of latent HIV-1 cellular reservoirs of infected individuals on virally suppressive HAART. The Journal of clinical investigation 115(1):128-37.

Weiss CD, Levy JA, White JM. 1990. Oligomeric organization of gp120 on infectious human immunodeficiency virus type 1 particles. Journal of virology 64(11):5674-7.

Wilson CC, Newman MJ, Livingston BD, MaWhinney S, Forster JE, Scott J, Schooley RT, Benson CA. 2008. Clinical phase 1 testing of the safety and immunogenicity of an epitope-based DNA vaccine in human immunodeficiency virus type 1-infected subjects receiving highly active antiretroviral therapy. Clinical and vaccine immunology : CVI 15(6):986-94.

Yu CI, Gallegos M, Marches F, Zurawski G, Ramilo O, Garcia-Sastre A, Banchereau J, Palucka AK. 2008. Broad influenza-specific CD8(+) T cell responses in humanized mice vaccinated with influenza virus vaccines. Blood 112(9):3671-3678.

Yu X, Matsuda Z, Yu QC, Lee TH, Essex M. 1993. Vpx of simian immunodeficiency virus is localized primarily outside the virus core in mature virions. Journal of virology 67(7):4386-90.

Zarocostas J. 2010. New HIV infections fall by a sixth in a decade. BMJ 341:c6739.

Zhu X, Ramos TV, Gras-Masse H, Kaplan BE, BenMohamed L. 2004. Lipopeptide epitopes extended by an Nepsilon-palmitoyl-lysine moiety increase uptake and maturation of dendritic cells through a Toll-like receptor-2 pathway and trigger a Th1-dependent protective immunity. European journal of immunology 34(11):3102-14.

Ziegler-Heitbrock L. 2007. The CD14+ CD16+ blood monocytes: their role in infection and inflammation. Journal of leukocyte biology 81(3):584-92.